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Interplay between the Specific Chaperone-Like Proteins HybG and HypC in Maturation of Hydrogenases 1, 2, and 3 from *Escherichia coli*

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The *hybG* gene product from *Escherichia coli* has been identified as a chaperone-like protein acting in the maturation of hydrogenases 1 and 2. It was shown that HybG forms a complex with the precursor of the large subunit of hydrogenase 2. As with HypC, which is the chaperone-like protein involved in hydrogenase 3 maturation, the N-terminal cysteine residue is crucial for complex formation. Introduction of a deletion into *hybG* abolished the generation of active hydrogenase 2 but only quantitatively reduced hydrogenase 1 activity since HypC could replace HybG in this function. In contrast, HybG could not take over the role of HypC in a Δ*hypC* genetic background. Overproduction of HybG, especially of the variants with the replaced N-terminal cysteine residue, strongly interfered with hydrogenase 3 maturation, apparently by titrating some other component(s) of the maturation machinery. The results indicate that the three hydrogenase isoenzymes not only are interacting at the functional level but are also interconnected during the maturation process.

Under anaerobic growth conditions, *Escherichia coli* is able to synthesize three [NiFe] hydrogenases, designated hydrogenase 1, 2, and 3 (2, 3, 28, 29). Hydrogenases 1 and 2 are uptake hydrogenases which couple H_2 oxidation to fumarate reduction, whereas hydrogenase 3 is a gas-evolving isoenzyme and an operational component of the formate hydrogenlyase complex (25, 28). The structural genes for hydrogenases 1 and 2 are located in the *hya* and *hyb* transcriptional units (22–24), whereas those coding for hydrogenase 3 are members of the *hyc* operon (5). Genes for a fourth hydrogenase (*hyf*) have been identified (1) but they do not appear to be expressed under the experimental conditions tested (27).

Apart from the structural genes, a set of seven genes has been identified whose products are involved in the synthesis and insertion of the [NiFe] metal center and maturation of the enzymes. Six of them were designated *hyp* (for hydrogenase pleiotropically acting genes) since mutations in most of them (*hypB*, *hypD*, *hypE*, and *hypF*) affect the maturation of all three hydrogenases (15, 18). The product of the seventh maturation gene is an endopeptidase which removes a C-terminal extension from each of the large subunits. As the cleavage reaction is a specific process, three different endopeptidases (HyaD, HybD, and HycI) are involved in the maturation of the large subunits of hydrogenases 1, 2, and 3, respectively (11, 24, 26).

When the genes coding for HypA and HypC were inactivated it was found that the mutations only affected the maturation of hydrogenase 3 (15). It was speculated that this apparent nonpleiotropic function could be due to the existence of two genes in the *hyb* operon, namely, *hybF* and *hybG*, whose derived amino acid sequences displayed similarity to those of *hypA* and *hypC*, respectively (15, 22). HybF thus may be the homolog of HypA and HybG may be that of HypC, and they could be involved in the maturation of hydrogenases 1 and 2.

The function of the *hypC* gene product has attracted considerable attention recently (10, 19). It was found that HypC is able to form a stable complex with the precursor of the large subunit (HycE) during the maturation process (10). The formation of the complex required the function of two residues, namely, the N-terminal cysteine residue of HypC (the N-terminal methionine is posttranslationally removed) and the first cysteine residue of the N-terminal motif (Cys241) involved in [NiFe] coordination (19). Since HypC forms a complex only with the precursor of the large subunit of hydrogenase 3 and not with the mature one, its function was tentatively envisioned as that of a specific chaperone, keeping the protein in a folding state amenable to metal insertion (10). Because the metal center of [NiFe] hydrogenases is located at the interface between the small and the large subunits (12, 30), an additional function of HypC could also reside in the prevention of the association during the maturation process (20). A putative catalytic role, however, is also possible.

In the present study, the function of HybG during formation of the three hydrogenase isoenzymes from *Escherichia coli* was analyzed. It is shown that HybG is the specific chaperone-like protein of hydrogenases 1 and 2 and that there is cross-interaction of the activities of HybG and HypC and also with other components of the hydrogenase maturation machinery.

MATERIALS AND METHODS

Bacterial strains, plasmids, and mutagenesis. The bacterial strains and plasmids employed in this study are listed in Table 1. *E. coli* DH5α was used as a host for plasmid construction and maintenance. The primers listed in Table 2 were used for the generation of plasmids carrying the *hybG* gene or derivatives thereof. For this purpose, the *hybG* gene was amplified via PCR with the aid of the primers HybF1 and YghU1 using chromosomal DNA of strain MC4100 as the template. The resulting PCR products were cloned into the *Eco*RV-restricted vectors pACYC184 and pBR322, leading to plasmids pAHBG and pBHBG, respectively. On these plasmids, the expression of the *hybG* gene is under the control of the *tet* promoter. To replace the cysteine residue of HybG with alanine and

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Reference
Strains		
DH5α	F$^-$ (ϕ80d*lacZ*Δ*M15*) *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)*U169*	32
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 ptsF25 deoC1 relA1 flbB5301 rpsL150* λ$^-$	7
HD705	MC4100 Δ*hycE*	27
HDK103	MC4100 Δ*hya* (Km^r) Δ*hycABCDEFGH*	15
HDK203	MC4100 Δ*hyb* (Km^r) Δ*hycABCDEFGH*	15
HDK200	MC4100 Δ*hyb* (Km^r)	M. Sauter (unpublished data)
DHP-C	MC4100 Δ*hypC*	15
NHC	DHP-C Δ*hybG*	This work
DHB-G	MC4100 Δ*hybG*	This work
CDH103	HDK103 Δ*hybG*	This work
Plasmids		
pMAK700	Cm^r	13
pACYC184	Cm^r Tc^r	8
pBR322	Ap^r Tc^r	6
pJA1021	pACYC184 *hypC* Cm^r	15
pJA16	pACYC184 *hypBCDE* Cm^r	18
pMΔHBG	pMAK700 with 225-bp deletion in *hybG*; Cm^r	This work
pAHBG	pACYC184 *hybG* Cm^r	This work
pAΔHBG	pACYC184 with 225-bp deletion in *hybG*; Cm^r	This work
pBHBG	pBR322 *hybG* Ap^r	This work
pBHBG-Strep	pBR322 *hybG-StreptagII* Ap^r	This work
pBC2A	pBR322 *hybG*[C2A] Ap^r	This work
pBC2S	pBR322 *hybG*[C2S] Ap^r	This work
pBC2A-Strep	pBR322 *hybG-StreptagII*[C2A] Ap^r	This work
pBC2S-Strep	pBR322 *hybG-StreptagII*[C2S] Ap^r	This work

serine, site-directed mutagenesis was performed on plasmid pBHBG as described previously (19) using the primer pairs HybGC2A-HybG1 and HybGC2S-HybG1, respectively. The fusion between the *hybG* gene and the sequence coding for the StreptagII was generated by PCR amplification of *hybG* on the plasmid pAHBG with the oligonucleotides HybF1 and HybG-StrepII. The resulting PCR product was then cloned into the *Eco*RV restriction site of pBR322.

The strains DHB-G (MC4100 Δ*hybG*), CDH103 (MC4100 Δ*hya* Δ*hyc* Δ*hybG*), and NHC (MC4100 Δ*hybG* Δ*hypC*) were constructed following the method of Hamilton et al. (13) with the aid of the pMAK plasmid system. An in-frame deletion of 225 bp was introduced into *hybG* by inverse PCR on pAHBG using the oligonucleotides HybG3′ and HybG5′ (Table 2), yielding the plasmid pAΔHBG. After restriction of pAΔHBG with *Bam*HI a 525-bp fragment, which includes the mutant *hybG* gene, was subcloned into the *Bam*HI-restricted pMAK700 vector. The resulting plasmid was designated pMΔHBG and was utilized to transfer the in-frame deletion onto the chromosome of the strains MC4100, HDK103, and DHP-C, leading to mutants DHB-G, CDH103, and NHC, respectively.

Growth conditions. *E. coli* cells were grown anaerobically at 37°C in a buffered rich medium, TGYEP (4), containing 15 mM sodium formate. As supplements, 1 μM sodium molybdate, 1 μM sodium selenite, and 5 μM nickel chloride were used. When needed for maintenance of plasmids or strains, the medium was supplemented with 30 μg of chloramphenicol, 50 μg of ampicillin, or 50 μg of kanamycin sulfate per ml. At an optical density at 600 nm of 1.0 the cells were harvested and stored at −20°C. The crude extracts were prepared as described previously (10).

Polyacrylamide gel electrophoresis and Western blotting. Proteins were separated on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels according to the method of Laemmli (17) or on 10% nondenaturating gels as specified by Drapal and Böck (10). Thirty micrograms of total protein was applied per lane.

For Western blot analysis, the proteins were transferred onto nitrocellulose membranes and the blots were reacted with antibodies raised against the large subunit of hydrogenase 2 (HybC) (1:1,500) or hydrogenase 3 (HycE) (1:1,000). The polyclonal antiserum directed against StreptagII was purchased from the Institut für Bioanalytik (Göttingen, Germany) and used at a dilution of 1:2,000.

To visualize the H_2-dependent benzyl viologen (BV) reduction activity, the nondenaturating gels were incubated in sodium phosphate buffer (100 mM, pH 7.2) containing 0.5 mM BV and 1 mM triphenyltetrazolium chloride in a glove box under an atmosphere of 95% N_2 and 5% H_2 for 24 h (3). One hundred micrograms of total protein was applied per lane.

RESULTS

HybG is required for maturation of hydrogenases 1 and 2. The final step of the maturation process of hydrogenases consists of the proteolytic removal of a C-terminal extension from

TABLE 2. Oligonucleotide primers used in this work

Designation	Sequence[a]
HybF1	5′-CAA**GGATCC**TGCGTTGAGGAGAGCGCCGT-3′
YghU1	5′-CGAACGTCCGGCGGTGAAACGTGG-3′
HybG-StrepII	5′-TCACTTTTCAAACTGAGGGTGGCTCCACGCGCTGGTAATG-3′
HybG3′	5′-TAT**GACGTC**TATACCAGCGCGTGATGA-3′
HybG5′	5′-ATA**GACGTC**ATAGCCAATACACATTAT-3′
HybGC2A	5′-GCCTGGAACGCCAATAGCCATTATTAACTCCGG-3′
HybGC2S	5′-GCCTGGAACGCCAATGCTCATTATTAACTCCGG-3′
HybG1	5′-ATTGGCGTTCCAGGCCAGGTGCTGGCTGTC-3′

[a] Bases changed in comparison to the wild-type sequence are underlined; newly generated restriction sites are shown in boldface type.

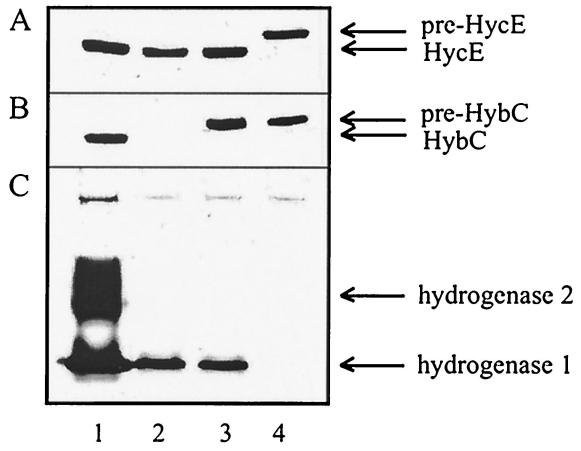

FIG. 1. Immunoblotting analysis of the precursor and mature forms of HycE and HybC in the wild-type strain MC4100 and in mutants with lesions in *hybG* and *hypC*. Crude extracts (30 μg protein) were subjected to SDS-polyacrylamide gel electrophoresis and reacted with antibodies raised against HycE (A) or HybC (B). Lane 1, MC4100; lane 2, HDK200 (Δ*hyb*); lane 3, DHB-G (Δ*hybG*); lane 4, NHC (Δ*hybG* Δ*hypC*) (lane 4). (C) Hydrogenase activity staining of the same strains is displayed after nondenaturating polyacrylamide gel electrophoresis.

the precursor of the large subunit. The assessment of the processing status, therefore, can be taken as a measure of the function of the components of the maturation machinery (10, 26).

To study the in vivo maturation role of HybG by this approach, its gene was inactivated by the introduction of an in-frame deletion, yielding strain DHB-G. The Δ*hybG* lesion leads to the blockage of the maturation of the hydrogenase 2 (pre-HybC) without affecting processing of hydrogenase 3 (pre-HycE) (Fig. 1A and B, lanes 3). In the Δ*hybG* Δ*hypC* double mutant (NHC) (Fig. 1A and B, lanes 4) neither pre-HybC nor pre-HycE is processed.

In order to analyze the influence of *hybG* or *hypC* deletions on hydrogenase isoenzymes 1 and 2, cell extracts were separated by nondenaturing polyacrylamide gel electrophoresis and the gels were analyzed by hydrogenase activity staining (Fig. 1C). Under these conditions, hydrogenase 3 activity cannot be detected as previously reported (28). The results indicate that the *hybG* deletion abolishes hydrogenase 2 activity but not that of hydrogenase 1. Albeit reduced, its activity is on the order of the level displayed by a mutant (HDK200) carrying a deletion within the *hyb* operon.

The finding that there was still hydrogenase 1 activity present when the *hyb* operon was deleted or when *hybG* was inactivated suggested the involvement either of HypC or of some other unknown homolog in the maturation process. To follow this assumption, the NHC strain was also analyzed by hydrogenase activity staining (Fig. 1C, lane 4); the results show that deletion of both *hybG* and *hypC* completely abolished hydrogenase activity. Consequently, the hydrogenase 1 activity displayed by the Δ*hyb* or Δ*hybG* strains is most likely due to the presence of the HypC protein.

Since both HybG and HypC are able to participate in maturation of hydrogenase 1, it was important to study whether they also interact in the maturation of hydrogenases 2 and 3.

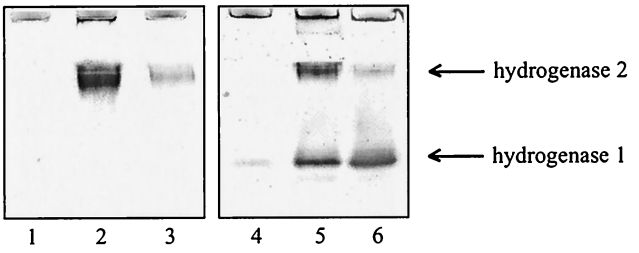

FIG. 2. Hydrogenase activity analysis of crude extracts of the Δ*hybG* strains CDH103 and DHB-G transformed with plasmids carrying *hybG* and *hypC* genes, respectively. Crude extracts (100 μg of protein) were subjected to nondenaturating polyacrylamide gel electrophoresis and stained for H_2-dependent BV reduction activity as indicated in Materials and Methods. Lane 1, CDH103; lane 2, CDH103/pAHBG (*hybG*); lane 3, CDH103/pJA1021 (*hypC*); lane 4, DHB-G; lane 5, DHB-G/pAHBG (*hybG*); lane 6, DHB-G/pJA1021 (*hypC*).

For this purpose, the two strains CDH103 (Δ*hya* Δ*hyc* Δ*hybG*) and DHB-G (Δ*hybG*) were transformed with a plasmid carrying either *hybG* (pAHBG) or *hypC* (pJA1021), and the transformants were analyzed for restoration of hydrogenase activity (Fig. 2). It was found that expression of *hybG* from the plasmid led to full restoration of the activity of hydrogenase 2 (Fig. 2, lanes 2 and 5) and to an increase of the activity of isoenzyme 1 (Fig. 2, lane 5). Supply of *hypC* in *trans* resulted in an increase of both hydrogenase 1 and 2 activities (Fig. 2, lanes 3 and 6). Conversely, to analyze whether pre-HycE can be matured by HybG in the absence of HypC, the Δ*hypC* mutant (DHP-C) was transformed with plasmid pAHBG, and the transformants were analyzed by immunoblotting (Fig. 3A, lane 5). It is evident that no processing took place when *hybG* was expressed in *trans*. Intriguingly, however, expression of *hypC* in *trans* as a control was unable to fully complement the *hypC* mutation (Fig. 3A, lane 4; see below). The results support the contention that the predominant role of HybG resides in the maturation

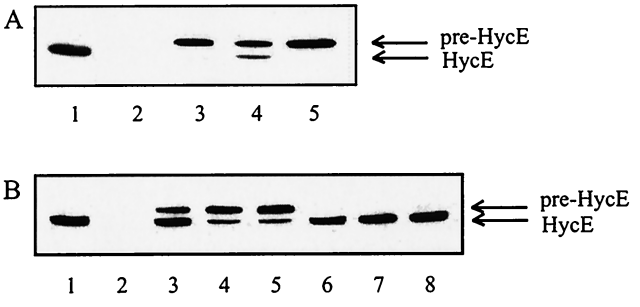

FIG. 3. Immunoblotting analysis of the precursor and mature forms of HycE. Crude extracts (30 μg protein) were subjected to SDS-polyacrylamide gel electrophoresis and reacted with antibodies raised against HycE. (A) Transformants of the Δ*hypC* strain (DHP-C) expressing either *hypC* or *hybG* genes from a plasmid were analyzed. Lane 1, MC4100; lane 2, HD705; lane 3, DHP-C; lane 4, DHP-C/pJA1021 (*hypC*); lane 5, DHP-C/pAHBG (*hybG*). (B) Transformants of MC4100 expressing *hypC* and different *hybG* variants from a plasmid were tested. Lane 1, MC4100; lane 2, HD705; lane 3, MC4100/pBHBG (*hybG*); lane 4, MC4100/pBC2A (*hybG*[C2A]); lane 5, MC4100/pBC2S (*hybG*[C2S]); lane 6, MC4100/pBHBG/pJA16 (*hybG hypBCDE*); lane 7, MC4100/pJA16 (*hypBCDE*); lane 8, MC4100/pJA1021 (*hypC*).

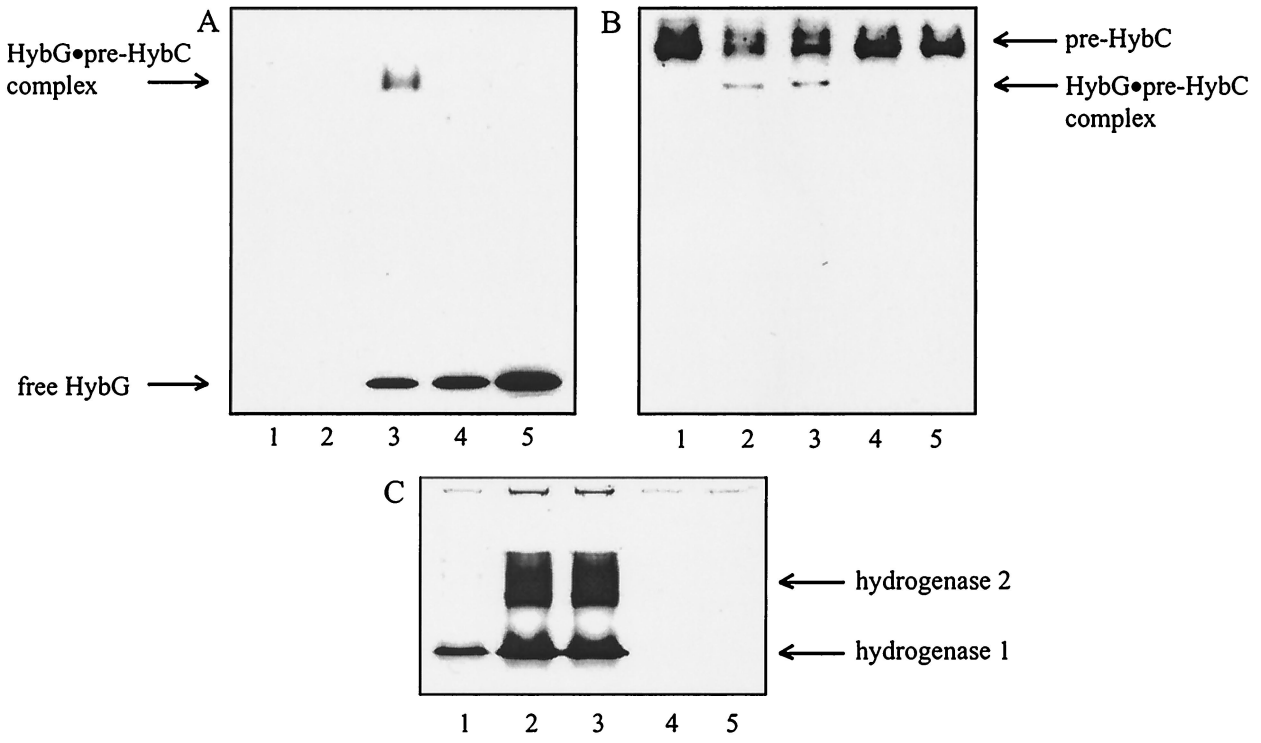

FIG. 4. Immunoblotting analysis of HybG–pre-HybC complex formation in transformants expressing different HybG variants from a plasmid. (A and B) Crude extracts (30 μg protein) were analyzed by Western blotting after nondenaturating polyacrylamide gel electrophoresis with antisera directed against StreptagII (A) and HybC (B). (C) Corresponding hydrogenase activity staining. Lanes 1, DHB-G; lanes 2, DHB-G/pBHBG; lanes 3, DHB-G/pBHBG-Strep; lanes 4, DHB-G/pBC2A-Strep; lanes 5, DHB-G/pBC2S-Strep.

of pre-HybC and that maturation of isoenzyme 1 can be supported by both HybG and HypC.

Overproduction of HybG interferes with the activity of HypC in maturation of hydrogenase 3. Overproduction of HypC in the Δ*hypC* genetic background did not fully substitute for the function of a *hypC* copy located at its indigenous chromosomal site (Fig. 3A, lane 4). This unexpected but intriguing finding could be the consequence of a polarity effect of the mutation on the expression of some downstream gene(s) or the effect of titration of some other component(s) of the maturation machinery. To differentiate between these possibilities, strain MC4100 was transformed with plasmids carrying either *hypC, hybG*, or one of the *hybG* variants constructed (see below), and processing of pre-HycE was studied by immunoblotting (Fig. 3B). It is evident that expression of *hybG* in *trans* impeded pre-HycE processing (Fig. 3B, lane 3) and the inhibition was augmented when the N-terminal cysteine of HybG was replaced by alanine or serine (Fig. 3B, lanes 4 and 5). Introduction of a second plasmid carrying the *hypBCDE* genes counteracted the inhibition exerted by the overproduction of HybG (Fig. 3B, lane 6). In contrast, expression of *hypC* from a plasmid did not interfere with pre-HycE maturation when the chromosomal *hypC* gene copy was present (Fig. 3B, lane 8).

HybG forms a complex with pre-HybC. To analyze whether HybG indeed has a chaperone-like function in the maturation of the large subunit of hydrogenase 2, its ability to form a stable complex with pre-HybC was studied. Since antibodies directed against HybG were not available, a fusion between *hybG* at its 3′ end and the StreptagII sequence was constructed, and a commercially available anti-StreptagII serum was used to localize the gene fusion product in nondenaturating polyacrylamide gels (Fig. 4). A HybG–pre-HybC complex could be detected both by anti-StreptagII antibodies (Fig. 4A, lane 3) and by anti-HybC antibodies (Fig. 4B, lanes 2 and 3). HybG was unable to enter the complex when its N-terminal cysteine was replaced by an alanine or serine residue (Fig. 4A and B, lanes 4 and 5). These HybG variants were also unable to generate hydrogenase 1 and 2 activities (Fig. 4C, lanes 4 and 5).

DISCUSSION

The complex between HypC and the precursor of the large subunit of hydrogenase 3 has been demonstrated to be the key intermediate in the maturation process (10). Its formation is an early step since mutants defective in each other gene involved in maturation accumulate the complex (10) and since its dissolution only precedes the final step, namely, endoproteolytic removal of the C-terminal extension of pre-HycE (20). In view of the high sequence similarity between HypC and HybG (~77%), a similar function was assumed for HybG in the maturation of hydrogenase 2 and possibly also of isoenzyme 1 (15, 22).

The in silico predictions now have been proven biochemically: HybG forms a complex with the precursor of the large subunit of hydrogenase 2 and as with HypC the N-terminal cysteine residue appears to be directly involved in complex formation. It is still open whether this residue solely has a structural role in the interaction of the two proteins or whether

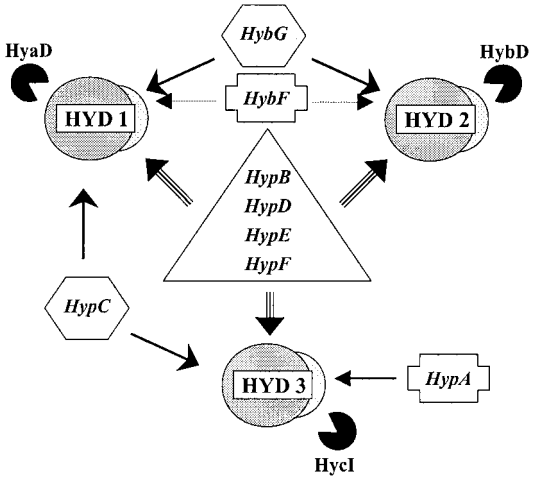

FIG. 5. Network of involvement of the auxiliary proteins in the formation of hydrogenases 1, 2, and 3. Dotted lines leading away from HybF indicate postulated functions.

its role is catalytic, e.g., in some redox reaction taking place during insertion of the metal(s) or the addition of the CO or CN ligands identified in the active site of hydrogenases (9, 14, 31).

Intriguingly, HybG has a dual function in that it is also required for maturation of hydrogenase 1. However, whereas the function of HybG is indispensable for the maturation of hydrogenase 2, its involvement in hydrogenase 1 maturation can be partially taken over by HypC. The evidence is that a Δ*hybG* mutant contains significant levels of hydrogenase 1 activity which can be augmented by overproducing HypC in *trans*. Also, a double mutant lacking both HybG and HypC is unable to form the three hydrogenase isoenzymes in a processed and active form.

The specificity and the interactions of the various maturation components are summarized in Fig. 5. HypB, HypD, HypE, and HypF are required for the synthesis of all three hydrogenase isoenzymes, since they are possibly involved in general reactions like nickel acquisition and donation (21) or CO-CN ligand biosynthesis. Others, such as HybG and HypC, are shared between two of the systems or they are specific like the endopeptidases (HyaD, HybD, and HycI), processing the precursors of the large subunits after the metals have been inserted. It will be interesting to study the consequences of these interconnections under different physiological conditions.

The results of this study are also relevant for the discussion of the physiological role of the three hydrogenase isoenzymes. Whereas the function of hydrogenase 2 as an uptake enzyme and that of hydrogenase 3 as a fermentative gas-evolving one are undisputed, that of hydrogenase 1 is not fully understood (3, 16). If it is indeed H_2 recycling the results would add another argument for such a role since the two enzymes would not only be interacting functionally but also be connected via a common component during the maturation process.

When overexpressed in *trans, hybG* interfered with hydrogenase 3 maturation in an otherwise wild-type genetic background: the inhibition was particularly pronounced in the case of the HybG variants in which the N-terminal cysteine was altered. A plausible explanation is that the overproduced HybG sequesters some other component(s) of the processing machinery, thereby forming unproductive processing intermediates in the case of the N-terminally altered variants. The fact that coexpression of the *hypBCDE* genes alleviated the inhibition by the HybG variants provides support for this contention.

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