The Auxiliary Protein HypX Provides Oxygen Tolerance to the Soluble [NiFe]-Hydrogenase of Ralstonia eutropha H16 by Way of a Cyanide Ligand to Nickel*

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The hypX gene of the facultative lithoautotrophic bacterium Ralstonia eutropha is part of a cassette of accessory genes (the hyp cluster) required for the proper assembly of the active site of the [NiFe]-hydrogenases in the bacterium. A deletion of the hypX gene led to a severe growth retardation under lithoautotrophic conditions with 5 or 15% oxygen, when the growth was dependent on the activity of the soluble NADH-dependent hydrogenase. The enzymatic and infrared spectral properties of the soluble hydrogenase purified from a hypX-negative strain were compared with those from an enzyme purified from a hypX-positive strain. In activity assays under anaerobic conditions both enzyme preparations behaved the same. Under aerobic conditions, however, the mutant enzyme became irreversibly inactivated during H₂ oxidation with NAD⁺ or benzyl viologen as the electron acceptor. Infrared spectra and chemical determination of cyanide showed that one of the four cyanide groups in the wild-type enzyme was missing in the mutant enzyme. The data are consistent with the proposal that the HypX protein is specifically involved in the biosynthetic pathway that delivers the nickel-bound cyanide. The data support the proposal that this cyanide is crucial for the enzyme function under aerobic conditions.

Hydrogenases (reaction H₂ ↔ H⁻ + H⁺ ↔ 2H⁺ + 2e⁻) play an important role in microbial energy metabolism involving molecular hydrogen. Three classes of hydrogenases can be distinguished according to the metal content of the H₂-activating site: [NiFe]-hydrogenases, [FeFe]-hydrogenases (previously called [Fe]-hydrogenases or Fe-only hydrogenases) and [Fe]-hydrogenases (1–4). Sequence comparisons, structural data, and spectroscopic properties indicate that although these hydrogenases are phylogenetically unrelated, they possess some remarkable similarities in the molecular architecture of the active site, namely the presence of cyanide and/or carbon monoxide as ligands to the metals. [FeFe]-hydrogenases are restricted to anaerobic bacteria and lower eukaryotes, whereas [NiFe]-hydrogenases are the dominant hydrogenases in Archaea and bacteria, including aerobic organisms. The [Fe]-hydrogenases (H₂-forming methylenetetrahydromethanopterin dehydrogenase) are found in many methanogenic Archaea (6).

In their active state the [FeFe]-hydrogenases are rapidly and irreversibly denatured by oxygen (7). Most [NiFe]-hydrogenases are reversibly inactivated by oxygen, whereas the [Fe]-hydrogenases are not affected by oxygen (3). Most hydrogenases are inhibited by carbon monoxide. A few [NiFe]-hydrogenases can function in air and are insensitive to carbon monoxide. Such O₂- and CO-insensitive [NiFe]-hydrogenases are of great biotechnological interest in relation to their potential use in fuel cells (8).

The best studied example of this group is the cytoplasmic soluble NAD⁺-reducing [NiFe]-hydrogenase (SH)² from the β-proteobacterium Ralstonia eutropha H16. This is a heterotetrameric enzyme with subunits HoxF (67 kDa), HoxH (55 kDa), HoxU (26 kDa), and HoxY (23 kDa) (9, 10). The enzyme comprises two functionally different heterodimeric complexes, which have been separated and characterized (9, 11). The HoxFU dimer constitutes an enzyme module, termed NADH dehydrogenase or diaphorase, involved in the reduction of NAD⁺ and holds one FMN group (called FMN-a) and several Fe-S clusters. The HoxHY dimer forms the hydrogenase module and contains a second functional FMN group (FMN-a) (12).

All [NiFe]-hydrogenases minimally consist of two subunits of different size (1, 2, 13). The larger subunit accommodates the active NiFe site. The smaller subunit contains at least one [4Fe-4S] cluster, called the proximal cluster as it is situated close to the active site. In many enzymes the latter subunit harbors two more clusters. The Desulfurobrio gigas enzyme contains a second [4Fe-4S] cluster (distal) and a [3Fe-4S] cluster (medial) situated between the two [4Fe-4S] clusters (14, 15). The aerobically purified standard [NiFe]-hydrogenases are inactive. Their active site is a (R,S)₅Ni(μ-R,S)₅(μ-OFe(CN))₃(CO) center (R = Cys) (14–23) and is depicted in Fig. 1A. The bridging oxygen ligand is removed upon reduction, whereby these enzymes become activated (17, 24).

The SH of R. eutropha belongs to a subclass of [NiFe]-hydrogenases where the polypeptide of the small hydrogenase subunit (HoxY) ends shortly after the position of the fourth Cys residue coordinating the proximal cluster. The large hydrogenase subunit (HoxH) of the SH contains the four Cys residues, conserved in all [NiFe]-hydrogenases, for the binding of the

2 The abbreviations used are: SH, soluble NAD⁺-reducing hydrogenase; BV, benzyl viologen.
bacterium, and Aquifex aeolicus, a hyperthermophilic bacterium. One particular feature, however, is common to all the eight species. They are strictly respiratory, preferentially aerobic, organisms. So far, the hypX gene has not been found in strictly anaerobic organisms. Deletion of hypX led to a complete knock-out of hydrogenase activity in R. leguminosarum (31) and decreased the MBH and SH activity of R. eutropha by ~50% (32). The H₂ sensor in R. eutropha remained unaffected (33).

The present study describes that hypX-mutant cells of R. eutropha showed a retarded growth on H₂ under standard aerobic conditions, demonstrating that the oxygen tolerance of the SH requires the function of HypX. The purified SH from the hypX-deletion strain did not contain the nickel-bound CN present in enzyme from HypX-containing cells, suggesting that this CN ligand is important for the oxygen tolerance.

**EXPERIMENTAL PROCEDURES**

**Construction of R. eutropha HF480**—The mobilizable plasmid pCH630 containing hypΔX was transferred from E. coli SI7-1 to R. eutropha HF359 (poxGΔ) by a spot mating technique (34). Gene replacement in R. eutropha was achieved by using an allelic exchange procedure based on the conditionally lethal sacB gene (35). The resulting isolates were screened for the presence of the desired deletion in hypX by PCR amplification of the respective target site (36). Deletion-carrying isolates were identified on the basis of the altered electrophoretic mobility of the amplification products. The resulting strain was named HF480 (poxGΔ hypΔX) (see summary in Table I).

**Cell Growth**—For lithoautotrophic growth, R. eutropha strains were cultivated in minimal medium (37) under an atmosphere of H₂, O₂, and CO₂ as specified under “Results.” For the isolation of the SH, R. eutropha strains were heterotrophically grown in fructose-glycerol minimal medium (37) in a 50-liter BIOSTAT D fermentor (Braun, Melsungen, Germany) at 30 °C under hydrogenase-derepressing conditions. The cells were harvested at an OD₆₀₀ of 11, washed with potassium phosphate buffer (50 mM, pH 7.0), rapidly frozen in liquid nitrogen, and stored at ~70 °C.

**Purification of the SH**—Cell-free extracts from R. eutropha cells (45-g wet weight) were prepared as described earlier (38). The SH was obtained by fractionation of (NH₄)₂SO₄ precipitation (35–60% saturation) followed by ion-exchange chromatography on a DEAE-Sephaloc column (39). The subsequent protocol was modified as follows. Fractions with high NAD⁺-reducing activity derived from the DEAE column were pooled and concentrated by (NH₄)₂SO₄ precipitation (60% saturation). Precipitated proteins were resuspended in 3 ml of potassium phosphate buffer (200 mM, pH 7.0), and the sample was applied to a phenyl-Sepharose-6 fast-flow HP column (5 × 100 mm, Amersham Biosciences). The column was washed with potassium phosphate buffer (pH 7.0), two bed volumes of 200 mM and two volumes of 50 mM. The enzyme was eluted with three volumes of 10 mM of the same buffer. Fractions of 6-ml were collected, and the homogeneity of the SH in fractions with high NAD⁺-reducing activity was further analyzed by SDS-PAGE with subsequent Coomassie staining. Fractions with an appropriate homogeneity were combined and concentrated by ultrafiltration (Centriprep 50, Millipore). The sample was rapidly frozen in liquid nitrogen and stored at ~70 °C. In the rest of the SH, the purified strain from HF359 (poxGΔ) was named SH(hypX+), and the SH purified from strain HF480 (poxGΔ hypΔX) is named SH(hypX−).

**Activity Measurements**—Routine hydrogenase activities and the influence of O₂ were measured at 30 °C in a 2.1-ml cell with a Clark electrode (type YSI 5323) for the polarographic measurement of H₂ (40). For activity under aerobic conditions, the cell was filled with aerobic buffer (50 mM Tris-HCl, pH 8.0, 5–10 μl of enzyme, and H₂-saturated water to a final H₂ concentration of 36 μM). Subsequently, NADH (5 μM) was added to activate the enzyme, followed by either benzyl viologen (BV) (2.5 mM) or NAD⁺ (5.0 mM). When anaerobic conditions were required, all solutions were flushed with argon before use, and glucose (50 mM) plus glucose oxidase (9 units/ml) were added to the reaction medium 3 min before the NADH addition. This minimized interference of oxygen.

To measure the CO sensitivity of the enzyme, activity was measured spectrophotometrically at room temperature. The assay buffer (50 mM Tris-HCl, pH 8.0) was flushed with H₂ gas. To 2 ml of this buffer in a septum-sealed cuvette, enzyme (~10 μM) was added plus a small
amount of NADH (5 μM) to activate the enzyme. Subsequently, 0.5 ml of argon- or CO-flushed buffer was added, after which argon-flushed solutions of either BV (4.2 mM, ε = 25 mV·cm⁻¹·mol⁻¹) or the physiological redox partner NAD⁺ (1.0 mM) was supplied as electron acceptors. NADH formation was measured with a Zeiss M4 QII spectrophotometer, monitoring the increase in absorption at 340 nm (ε = 6.22 mV·cm⁻¹·mol⁻¹). Reduction of BV was monitored at 640 nm, rather than on its absorbance maximum (ε = 10,000 mV·cm⁻¹·mol⁻¹ at 555 nm). Before use, H₂ was passed over a palladium catalyst (Degussa, Hanau, Germany; type E236P), and argon was passed through an Oxisorb cartridge (Messer-Griesheim, Düsseldorf, Germany) to remove residual O₂.

Fourier-transform Infrared Spectra—Fourier-transform infrared spectra were collected as before (23). A fitting of infrared spectra was performed with a minimal set of Gaussian functions using GRAMS software (Galactic Ind. Corp.).

Miscellaneous—Protein concentrations were determined according to Bradford (41). The nickel and cyanide content of the preparations was determined as described (23). The spin concentration calculated from the EPR signal of the [2Fe-2S]²⁺ cluster in reduced enzyme (1 bar H₂, for 45 min at 30 °C) was used as a measure for the enzyme concentration (42).

RESULTS

Effect of Deletion of hypX on Lithoautotrophic Growth—The doubling time of R. eutropha H16 wild type under lithoautotrophic conditions is 3.8 h. The deletion of hypX led to a slight increase in the doubling time to 4.4 h (32). To investigate whether HypX is of physiological relevance to R. eutropha, and more specifically to the functioning of the soluble hydrogenase, we introduced a hypX deletion into a strain impaired in the MBH genes. Cells of the resulting double mutant were grown lithoautotrophically with CO₂ as the carbon source and H₂ as the energy source, in the presence of either 5 or 15% O₂. The SH-supported growth was severely affected by the hypX mutation in comparison to the HypX-containing control strain (Fig. 2). Thus, growth of the SH-containing HypX-deletion mutant was significantly delayed and only slowly started after a long lag phase (40–60 h) depending on the oxygen-partial pressure. The doubling time increased (Fig. 2) from 3.8 h in the control strain to 53 h (5% O₂) or 66 h (15% O₂). Normal oxygen-tolerant growth was restored by genetic complementation with hypX (data not shown). These experiments demonstrated that the absence of the HypX protein severely affects the physiological function of the soluble [NiFe]-hydrogenase under aerobic conditions.

Infrared Spectra of the SH Purified from the hypX-deletion Mutant—To investigate the SH that is from the hypX deletion strain in more detail, the SH was purified from cells of mutant HF480 (SH/HypX⁻) cultivated heterotrophically under hydrogenase-derepressing conditions. Purified SH(HypX⁻) was used as control in the following experiments.

Infrared spectra of the as-isolated enzyme preparations are shown in Fig. 3I. As expected from previous studies (26, 43), the oxidized SH(HypX⁺) enzyme (Fig. 3I, A) showed an intense absorption band at 1956 cm⁻¹ because of the stretching vibration of a CO coordinated to the active site iron. A reasonable fit of the peaks in the cyanide region of the spectrum (2120–2050 cm⁻¹) was obtained with four peaks at 2098 (18%), 2088 (28%), 2080 (32%), and 2070 (22%) cm⁻¹. Because of the asymmetry of the experimental band at 2080 cm⁻¹, the fit improved by adding a small band at 2077 cm⁻¹, resulting in a fit with five peaks at 2098 (18%), 2088 (30%), 2081 (20%), 2077 (12%), and 2070 (20%) cm⁻¹. The CN band at 2098 cm⁻¹ of the oxidized SH(HypX⁺) was absent in the spectrum of the SH(HypX⁻) mutant enzyme (Fig. 3I, B). A three-peak fit gave bands at 2087 (28%), 2081 (41%), and 2070 (31%) cm⁻¹. The asymmetry of the experimental band at 2081 cm⁻¹ was better reproduced by adding a small fourth peak at 2077 cm⁻¹, resulting in a fit with four peaks at 2087 (29%), 2081 (37%), 2077 (3%), and 2070 (31%) cm⁻¹. The positions and the relative intensities of these bands are very similar to those present in spectra of the control (Fig. 3I, A), except for the missing 2080 cm⁻¹ band. Treatment of the enzyme preparation with oxidizing agents like oxygen or 2,6-dichlorophenolindophenol did not alter this spectrum. A reduction of the SH(HypX⁻) during a redox titration did not result in shifts of any of the bands (Fig. 3II), whereas in wild-type enzyme the 2080 cm⁻¹ band shifted to 2090 cm⁻¹ at −316 mV, and subsequently to 2051 cm⁻¹ at −516 mV (not shown). Likewise, incubation under H₂ in the presence of 10 μM NADH did not induce any changes in the IR spectrum of SH(HypX⁻). These properties are consistent with the conclusion that the nickel-bound cyanide is missing in the SH purified from the hypX-mutant cells.

A characteristic feature of standard [NiFe]-hydrogenases is their sensitivity to CO. Carbon monoxide binds to nickel and competes with H₂ for binding at the active site (19, 44, 45). The SH activity is not inhibited by CO (39, 46, 47). To see whether

### Table 1

| Strain or plasmid | Relevant characteristics | Source or reference |
|-------------------|--------------------------|---------------------|
| R. eutropha       | wild type, SH⁺ MBH⁺ HypX⁺ | DSM428, ATCC 17699  |
| H16               | SH⁺ MBH⁺ HypX⁺ (λoxG2)   | This study          |
| HF359             | SH⁺ MBH⁺ HypX⁺ (λoxG2 hypX⁻) |
| HF480             | SH⁺ MBH⁺ HypX⁺ (λoxG2 hypXα) |
| E. coli           | Tra+ recA pro thi hsdR, chr- RP4-2 |
| S17-1             | Km⁺, sacB, RP4 oriT, ColE1 ori |
| Plasmids          |                           |                     |
| pLO2              | Km⁺, sacB, RP4 oriT, ColE1 ori |
| pCH630            | 1.3-kb NsiI fragment containing hypXΔ in pLO2 |

FIG. 2. Lithoautotrophic growth of the hypX-deletion strain under various oxygen-partial pressures. Shown are the SH-dependent growth curves of the MBH- strain HF359 (circles) and the MBH⁻, HypX⁻ strain HF480 (squares). The gas phase (1 bar) contained 10% CO₂ and 75% H₂. The remaining 15% consisted of 10% N₂ plus 5% O₂ (filled symbols) or of 15% O₂ (open symbols).
the SH(HypX+) enzyme behaves differently, the hydrogen-reduced sample was incubated with 1 bar of CO for 30 min. No differences in the infrared spectra were observed (data not shown). The lack of an extra peak originating from added CO indicates that it does not bind to the active site, neither in the wild-type enzyme nor in the SH(HypX+) mutant enzyme. This was in agreement with the lack of CO inhibition in activity measurements (data not shown).

Chemical Determination of Cyanide—To determine whether the lack of one absorption band assigned to the nickel-bound CN group in the SH(HypX+) enzyme (Fig. 3, B) correlates with a decreased level of cyanide, the mutant SH was subjected to a chemical analysis for cyanide (23). The SH(HypX+) enzyme contained 2.9 cyanides/nickel (determined by atomic-absorption spectrometry). As controls the SH from R. eutropha H16 and the standard [NiFe]-hydrogenase from Allochromatium vinosum were used, and those yielded values of 3.7 and 1.9 cyanides/nickel, respectively (26). This result with the SH(HypX+) enzyme is in accordance with the spectroscopic data and demonstrates that this mutant enzyme lacks the CN group bound to nickel.

Catalytic Properties of the SH(HypX+)—To examine whether the modification of the nickel-iron active site affected the catalytic properties of the mutant enzyme, the purified SH(HypX+) was subjected to a number of enzymatic assays. The results are summarized in Table II. Within error, the NADH dehydrogenase activity (measured as the oxidation of NADH by BV) of the SH(HypX+) was the same as that of the wild-type SH. This activity is mediated by the HoxHY module of the SH and is independent of the hydrogenase module HoxHY (11).

We experienced that with purified preparations of the wild-type SH the H2-acceptor oxidoreductase activities can be rather much less (e.g. see Refs. 12 and 26). Although the H2-oxidizing activities of the SH(HypX+) mutant enzyme using either NAD+ or BV as electron acceptor and the NADH-dependent H2 production activity were lower than those of the control enzyme (Table II), the values were within the range observed for routine SH preparations. The Km for hydrogen was the same for both enzymes. Thus, these catalytic properties were not seriously affected, if at all, by the deletion of the hypX gene. These data do not explain, however, why cells of the HypX− mutant show a severely retarded growth with hydrogen in the presence of oxygen (Fig. 2).

Effect of the hypX Mutation on the Oxygen Sensitivity of Purified SH—To compare the oxygen tolerance of the SH(HypX+) and the mutant SH(HypX−), amperometric H2-uptake measurements were performed. First we inspected the H2-dependent reduction of NAD+ of aerobically isolated non-activated SH samples (Fig. 4F). In aerobic buffer, the activity trace of the SH(HypX+) showed a lag phase, reflecting the process of the H2-dependent autocatalytic activation of the enzyme due to increasing amounts of NADH produced by a minor portion of active enzyme molecules. This autocatalytic behavior is well known (39). The maximum activity was obtained after ~2 min (Fig. 4I, A). A completely different shape of the activity trace was obtained with the SH(HypX+) (Fig. 4I, B). Although the activity slightly increased during the first few minutes, it subsequently became constant and decreased at longer times. At that time, addition of NADH as a specific reductant for the enzyme did not restore enzyme activity (data not shown), indicating that the H2-oxidizing capacity of the SH was irreversibly destroyed. Under anaerobic conditions, the autocatalytic activation of the SH(HypX+) proceeded just like that of the SH(HypX+) (Fig. 4I, C and D). Thus, the inhibitory effect on the activity of SH(HypX−) in trace B was because of the presence of oxygen in that specific assay.

To examine whether O2 affected the catalytic turnover or only the autocatalytic activation process of SH(HypX−), a second set of experiments was carried out in which the enzyme was activated by a reduction with NADH prior to the start of the reaction. Under these conditions, SH(HypX+) was as active as SH(HypX−), and activities were independent of the presence or absence of oxygen (Fig. 4II, A–D). Thus, at first glance, the turnover activity of SH(HypX+) did not seem to be affected by oxygen. The differences in behavior shown in Fig. 4, I and II are explained as follows. In the absence of NADH at the start of the reaction, NAD+ can be reduced only by a very small portion of the enzyme molecules that happen to be active. This results in the formation of minute amounts of NADH, which in turn activate other enzyme molecules. Any contaminating NADH in the used NAD+ will also facilitate this process. For the
SH(HypX) the presence of oxygen hardly affected this autocatalytic activation (Fig. 4, A and C). The SH(HypX)14 however, apparently reacted with oxygen in a way that prevented this activation and even resulted in the irreversible destruction of the enzyme (Fig. 4, A and C). When started in the presence of 10 μM NADH (Fig. 4, II), the NADH concentration will always be three orders of magnitude greater than that of the enzyme (present in nanomolar amounts). Any inactivation of the enzyme by oxygen will be counteracted by the specific reactivation by NADH. The H2 → NAD+15 activity under anaerobic conditions was always ~20% lower than the activity under anaerobic conditions. This is indicative of some inactivation (and immediate re-activation by NADH) influencing the overall activity, albeit to only a small extent.

A third set of experiments was conducted using the artificial electron acceptor BV (Fig. 4III). Also in this case the enzyme was activated by addition of 10 μM NADH just before the addition of BV. The SH(HypX)16 showed a high level of activity under both aerobic and anaerobic conditions (Fig. 4III, A and C). In contrast, the initial high activity observed for SH(HypX)17 in an aerobic buffer stopped within 1 min, although plenty of H2 was still present (Fig. 4III, B). Activity could be partially restored by the addition of NADH, but after ~5 min, reactivation with NADH was no longer possible, indicating that enzyme activity was irreversibly destroyed at that time point (data not shown). Under anaerobic conditions the SH(HypX)18 was as active as the SH(HypX)19, and no inactivation was observed (Fig. 4III, D). This is explained as follows. Under aerobic conditions the produced reduced BV is immediately auto-oxidized by O2 and is therefore not available for enzyme reduction and reactivation. In addition, the NADH dehydrogenase module catalyzes the oxidation of NADH by BV. After consumption of the NADH, the control enzyme remained active (no inactivation by O2), but the SH(HypX)10 stopped its action because of its sensitivity toward O2 (Fig. 4III, B).

**DISCUSSION**

The Active Site of the SH(HypX)11—Based on the enzymatic, infrared-spectroscopic, and chemical analyses results presented in this paper, we conclude that the active site of the SH(HypX)11 lacks the nickel-bound cyanide detected in the SH(HypX)12 (Fig. 1B). As a result, the enzyme becomes highly sensitive to oxygen inactivation during turnover in the presence of low concentrations of NADH. When NADH is present in excess to the enzyme concentration, the SH(HypX)13 is protected against this inactivation. The turnover capacity under anaerobic conditions was not affected. The properties of the SH(HypX)14 are highly similar to those of an SH preparation where the nickel-bound cyanide was removed by prolonged reduction of the SH under H2 (26). These findings are in agreement with the earlier proposal that the nickel-bound cyanide plus the third cyanide bound to iron protect the active site, by steric hindrance, from an attack by oxygen under turnover conditions (26, 43).

The active site in the intact SH is not completely protected against O2. The as-isolated SH is inactive and requires a reductive activation by NADH. It is hypothesized that the oxygen species (Fig. 1B) is thereby removed so that H2 can bind to nickel to become heterolytically split. When the reduced active enzyme is reoxidized under anaerobic conditions it remains in the active state in air (48). However, when reduced active SH is reoxidized by air it is (reversibly) inactivated. Thus, the inactivation of the SH by O2 occurs only when the enzyme contains reducing equivalents (49, 50). This suggests that an oxygen-reduction product (hydroxide or peroxide) may be bound to nickel. The formation of some superoxide cannot be excluded. We propose that the absence of the nickel-bound cyanide greatly enhances the rate of reaction of the reduced SH(HypX)16 with oxygen, thereby rapidly converting the enzyme to the inactive state. It is anticipated that the amount of superoxide produced/time unit will also increase substantially and that this will presumably result in the enhanced destruction of the active site. This is how we explained the delay in the autocatalytic activation (Fig. 4, B), the inactivation during the reaction with BV (Fig. 4III, B), and, at longer times, the irreversible loss of activity. The presence of excess NADH will counteract this by the continuous reduction of the peroxide/superoxide produced at the location of the active site.

Standard [NiFe]-hydrogenases contain two cyanides in their active sites, both coordinated to the iron (Fig. 1A) and are instantaneously (reversibly) inactivated when O2 or CO are introduced during turnover (5). The properties of the SH(HypX)17 show that even in the absence of the nickel-bound cyanide the active site is not reacting with external CO. It is anticipated that the extra cyanide on iron in the SH active site is mainly responsible for the protection against CO binding, although the mechanism of this protection remains elusive.

**Effect of the Absence of HypX on Lithoautotrophic Growth—** Fig. 2 shows that the deletion of the hypX gene nearly eliminated the SH-dependent growth in the presence of 5% oxygen. In view of the enzymatic, infrared-spectroscopic, and chemical analyses of the SH(HypX)18 described above, this effect can be explained as follows. During growth on H2 and O2, the bacterium is forced to use the SH for the acquisition of reducing equivalents from H2. The enzyme converts these reducing equivalents into NADH. Subsequently this is oxidized by NADH:ubiquinone oxidoreductase, and the rest of the respiratory chain for the generation of a proton gradient. In addition, the CO2-fixation process in autotrophically growing cells consumes large amounts of NADH. This means that the steady-
state concentration of NADH in lithoautotrophically growing cells will be quite low. For the SH, these operating conditions are comparable with those in Fig. 4f. The aerobic autocatalytic activation of the intact SH already proceeds at very low NADH concentrations. Although oxygen can slowly react with the enzyme, the autocatalytic reductive activation of the enzyme prevailed. However, the SH(HypX) did not succeed in becoming active under these conditions because of the enhanced reaction with oxygen. The experiments also showed an irreversible inactivation of the enzyme at longer times (Fig. 4f, B).

The Function of the HypX Accessory Protein in the Biosynthesis of the Active Site—Two conserved domains have been identified within the HypX amino acid sequences, a N-terminal \(\text{N}^{10}\)-formyltetrahydrofolate-binding motif and an enoyl-CoA hydratase/isomerase domain in the C-terminal region (31). From these observations the authors suggested that HypX might have a function in the synthesis of the diatomic ligands CO and/or CN attached to the iron of the Ni-Fe active site. The present study demonstrated that the biosynthesis of the Fe(CN)\(_3\)(CO) group in the SH was not affected by the deletion of HypX but that the nickel-bound cyanide was specifically missing. Hence we concluded that HypX is involved in the synthesis and/or delivery of the nickel-bound cyanide of the SH in a step, following the incorporation of both the Fe(CN)\(_3\)(CO) group and the Ni. This is consistent with the conserved \(\text{N}^{10}\)-formyltetrahydrofolate-binding domain in HypX pointing to a functional role in the synthesis of the Active Site—

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