CO synthesized from the central one-carbon pool as source for the iron carbonyl in O_2-tolerant [NiFe]-hydrogenase

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Hydrogenases are nature's key catalysts involved in both microbial consumption and production of molecular hydrogen. H_2 exhibits a strongly bonded, almost inert electron pair and requires transition metals for activation. Consequently, all hydrogenases are metalloenzymes that contain at least one iron atom in the catalytic center. For appropriate interaction with H_2, the iron moiety demands for a sophisticated coordination environment that cannot be provided just by standard amino acids. This dilemma has been overcome by the introduction of unprecedented chemistry—that is, by ligating the iron with carbon monoxide (CO) and cyanide (or equivalent) groups. These ligands are both unprecedented in microbial metabolism and, in their free form, highly toxic to living organisms. Therefore, the formation of the diatomic ligands relies on dedicated biosynthesis pathways. So far, biosynthesis of the CO ligand in [NiFe]-hydrogenases was unknown. Here we show that the aerobic H_2 oxidizer *Ralstonia eutropha*, which produces active [NiFe]-hydrogenases in the presence of O_2, employs the auxiliary protein HypX (hydrogenase pleiotropic maturation X) for CO ligand formation. Using genetic engineering and isotope labeling experiments in combination with infrared spectroscopic investigations, we demonstrate that the α-carbon of glycine ends up in the CO ligand of [NiFe]-hydrogenase. The α-carbon of glycine is a building block of the central one-carbon metabolism intermediate, N^{10}-formyl-tetrahydrofolate (N^{10}-CHO-THF). Evidence is presented that the multidomain protein, HypX, converts the formyl group of N^{10}-CHO-THF into water and CO, thereby providing the carbonyl ligand for hydrogenase. This study contributes insights into microbial biosynthesis of metal carbonyls involving toxic intermediates.

hydrogenase | metalloenzyme | carbonyl ligand | formyl-THF | one-carbon metabolism

Hydrogenases are abundant metalloenzymes in prokaryotes and lower eukaryotes in which they catalyze the reversible oxidation of molecular hydrogen into protons and electrons. Depending on the physiological conditions, hydrogenases enable their hosts either to use hydrogen as an energy source or to dissipate excess, reducing power as molecular hydrogen (1, 2). Enzymatic cycling of H_2 is characterized by high substrate specificity and high turnover rates and has received great attention from both fundamental and applied perspectives (3).

The two major classes of hydrogenases, [FeFe]- and [NiFe]-hydrogenases, are grouped on the basis of their metal content in the catalytic center. Although their active site structures differ considerably, the two hydrogenase types share uncommon, nonproteinaceous diatomic iron ligands. The diiron site of [FeFe]-hydrogenases is equipped with two cyanide (CN^-) and three carbon monoxide (CO) molecules, whereas the active site iron of [NiFe]-hydrogenases ligates two CN^- residues and one CO (1–5). Biosynthesis of these diatomic ligands involves intriguing chemistry, which is challenging for a living cell because of the toxicity of free CN^- and CO molecules. In the case of [NiFe]-hydrogenases, at least six conserved auxiliary proteins, designated HypA–F, are involved in the synthesis and incorporation of the $NiFe(CN)_2(CO)$ center into the apo-protein (4, 6). A complex of the HypD and HypC proteins acts as scaffold for the assembly of the $Fe(CN)_2(CO)$ entity of the active site (7, 8). The HypF and HypE proteins deliver the CN^- ligands, which are synthesized from carbamoyl phosphate (9). Incorporation of the nickel is facilitated by the HypB and HypA proteins (10). However, source and synthesis of the active site CO ligand remained elusive.

Maturation studies on the O_2-tolerant, energy-generating [NiFe]-hydrogenases in the facultative H_2-oxidizing bacterium *Ralstonia eutropha* H16 indicate that at least two different metabolic sources exist for CO ligand synthesis (11). Heterotrophic growth of *R. eutropha* with ^{13}C-glycerol as the sole source of carbon and energy led to a fully labeled CO ligand in hydrogenase, demonstrating that the carbonyl moiety originates from the cellular metabolism. Remarkably, selective removal of CO gas, which was released by *R. eutropha* cells during lithoautotrophic growth on H_2 and CO_2 in the presence of high O_2 concentrations, caused a considerable growth delay due to a reduced amount of fully maturated hydrogenase (11). Interestingly, a similar growth retardation was observed for a *R. eutropha* mutant deleted for the *hypX* gene (12). The *hypX* gene is a constituent of the *hyp* gene cluster in *R. eutropha* and occurs only in microbes synthesizing [NiFe]-hydrogenase under (micro)aerobic conditions (12). A number of potential functions, including nickel insertion (13), regulation (14), and cyanide ligand synthesis (15), had been assigned to the HypX protein, but none of them has been unambiguously validated.

Results and Discussion

We investigated the role of HypX in CO ligand biosynthesis during [NiFe]-hydrogenase maturation. Cells of the wild-type strain *R. eutropha* H16 and the *hypX*-deficient mutant HF469 (12)

Significance

Activation of dihydrogen is by far not a trivial catalytic reaction. Microbes have evolved sophisticated hydrogenases with complex transition metal centers to get access to H_2. A recurring feature of these centers is the presence of iron atoms equipped with carbon monoxide ligands. In case of [NiFe]-hydrogenases, which contain a $NiFe(CN)_2CO$ catalytic center, biosynthesis of the toxic CO ligand remained elusive. We show that [NiFe]-hydrogenases that are catalytically active in the presence of dioxygen use a dedicated maturase for CO ligand synthesis under aerobic conditions. CO is derived from the most oxidized intermediate of the central one-carbon metabolism, formyl-tetrahydrofolate. This discovery contributes a so far unknown reaction to the one-carbon metabolism and opens perspectives for chemical and of bioinspired catalysis.

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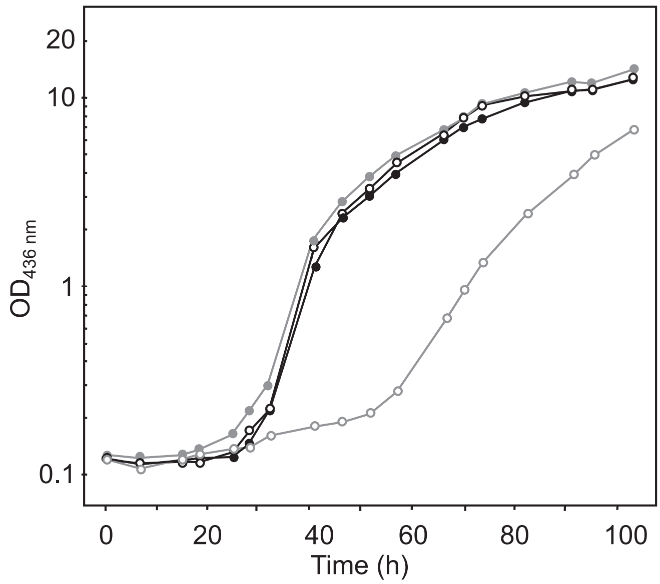

Fig. 1. Lithoautotrophic growth of the HypX-deficient *R. eutropha* derivative in the absence and presence of externally added carbon monoxide gas. Strains *R. eutropha* HF469 (Δ*hypX*, gray lines) and H16 (wild-type, black lines) were pregrown in fructose-ammonium (FN) minimal medium under hydrogenase-repressing conditions (37 °C). The precultures were used to inoculate the main cultures, which were grown in mineral salts medium without organic carbon source at 30 °C under a gas atmosphere composed of 10% H_2, 10% CO_2, 15% O_2, and 65% N_2 (all gas concentrations are given in vol/vol), either in the absence (open circles) or presence (closed circles) of 5,000 ppmv CO. The growth retardation of the Δ*hypX* strain was related to a diminished synthesis of the mature, energy-generating hydrogenases (Fig. S1).

were cultivated in mineral medium in the presence of H_2 as the sole energy source, CO_2 as the carbon source, and O_2 as the terminal electron acceptor, either in the absence or presence of externally added CO (Fig. 1). Compared with the wild-type strain, the Δ*hypX* mutant showed a considerable growth delay in the absence of exogenous CO gas, which was related to a diminished synthesis of the two energy-generating hydrogenases of *R. eutropha* (Fig. S1). Strikingly, addition of 5,000 ppmv CO to the headspace of the culture led to synthesis of fully maturated hydrogenase protein and enabled wild type-like growth of the Δ*hypX* mutant (Fig. 1 and Fig. S1). This supports the assumption of two independent pathways for CO ligand synthesis (11). At low cell density, correlating with exposure to high O_2 tension, HypX turns out to be crucial for biosynthesis of the carbonyl ligand.

Members of the HypX family display a conserved bipartite structure. The N-terminal part of the protein exhibits remarkable similarity to N^{10}-formyl-tetrathydrofolate (N^{10}-CHO-THF) hydrolases, whereas the C-terminal part resembles the structure of enoyl-CoA hydratase/isomerases of the crotonase superfamily (13). This observation raised the question of whether the formyl group of N^{10}-CHO-THF may act as precursor for biosynthesis of the carbonyl ligand. To verify this assumption, we designed an experimental setup based on specific labeling of the CO ligand with stable isotopes (Fig. 2).

THF-dependent one-carbon metabolism provides one-carbon units for fundamental biosynthetic processes such as methionine formylation and purine synthesis (16). According to the genetic inventory of *R. eutropha* H16 (17), two different pathways replenish the THF-dependent one-carbon pool. The first one involves serine hydroxymethyltransferase, which transfers the hydroxymethyl group from serine to THF, thereby producing 5,10-methylene-THF that, subsequently, is either oxidized stepwise to N^{10}-CHO-THF or reduced to methyl-THF (Fig. 2). The second pathway employs the glycine cleavage system, which produces 5,10-methylene-THF directly from glycine. As glycine biosynthesis relies on the serine hydroxymethyltransferase reaction, a strain lacking the corresponding enzyme (encoded by *glyA*) should be auxotrophic for glycine. Hence, isotopically labeled glycine appeared to be an ideal tool for tracing the carbonyl ligand in [NiFe]-hydrogenase. Following this strategy, we constructed an *R. eutropha* derivative

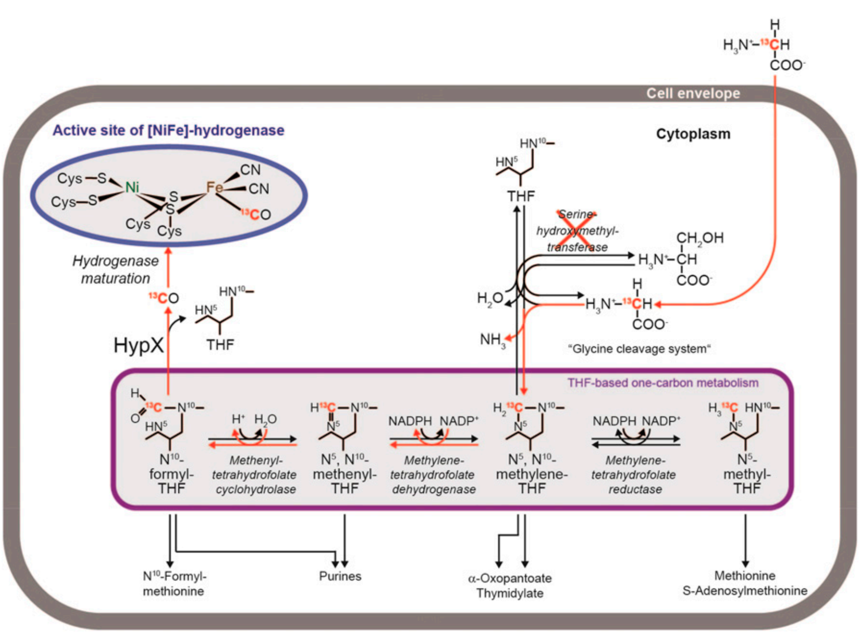

Fig. 2. Model of HypX-mediated biosynthesis of the CO ligand in [NiFe]-hydrogenase and glycine-derived CO labeling strategy. A knockout of the *glyA* gene encoding serine hydroxymethyltransferase causes glycine auxotrophy (Fig. S2). Under these conditions, externally added glycine, processed by the glycine cleavage system, serves as the sole source of one-carbon groups for the THF-based one-carbon metabolism. Red arrows indicate the proposed pathway of labeled 2-^{13}C-glycine toward the ^{13}CO ligand in the active site of [NiFe]-hydrogenases.

carrying an isogenic in-frame deletion in the *glyA* gene. As expected, the resulting auxotrophic mutant required glycine supplementation for growth (Fig. S2).

In a proof-of-concept experiment, we supplemented cells of the Δ*glyA* mutant with ^{13}C-glycine and analyzed the resulting labeling pattern of the purine derivative adenosine diphosphate (ADP) using mass spectrometry (MS). All glycine-derived carbon atoms were incorporated into ADP at their dedicated positions (Fig. S3), demonstrating that this experimental approach is reliable for selective labeling of N^{10}-CHO-THF.

To examine whether HypX uses a precursor of the THF-based one-carbon metabolism for synthesis of the carbonyl ligand in [NiFe]-hydrogenase, *R. eutropha* cells were grown aerobically under heterotrophic conditions in the presence of uniformly labeled ($^{13}C_2$) glycine. Of the four hydrogenases present in *R. eutropha* (18), we selected the regulatory [NiFe]-hydrogenase (RH) as the model system for our study. The RH undergoes a less complex maturation process than the other three hydrogenases (18); it can be conveniently purified from soluble cell extracts by affinity chromatography, and the carbonyl and cyanide ligands of the active site are easily accessible to IR spectroscopic analysis (11). An almost quantitative labeling of the carbonyl ligand was observed in the IR spectrum of the RH isolated from cells of the HypX$^+$ strain (Fig. 3*A*). This conclusion was drawn from the shift of the absorption band related to the iron-coordinated carbonyl group from 1,943 cm^{-1} (^{12}CO stretching vibration) (11) to 1,899 cm^{-1} (^{13}CO stretching vibration), which was not observed for an RH sample purified from the *hypX* knockout mutant (Fig. 3*B*). Notably, even in the presence of $^{13}C_2$-glycine, a minor portion of the CO ligands in RH protein isolated from the HypX$^+$ background remained unlabeled, indicated by a small band at the position characteristic for iron-coordinated ^{12}CO (Fig. 3*A*). This result in combination with the fact that the HypX-deficient strain revealed an almost complete absorption band shift to 1,943 cm^{-1} supports the coexistence of two independent pathways for CO ligand synthesis, as proposed in a previous study (11). Our current data clearly show that one of the two pathways relies on HypX as key factor.

To obtain further insight into HypX-driven CO ligand biosynthesis, experiments were conducted with differentially labeled glycine derivatives. The supply of 2-^{13}C-glycine yielded exactly the same labeling pattern of the RH as that obtained with uniformly labeled $^{13}C_2$ glycine (Fig. 3 *C* and *D*). By contrast, addition of 1-^{13}C-glycine did not cause any shift of the CO band in the HypX$^+$ background (Fig. 3*E*). This result demonstrates that the carbonyl ligand exclusively derives from the 2-^{13}C-atom of glycine and is consistent with the fact that the THF-based one-carbon pathway is replenished by the glycine cleavage system, allocating the 2-^{13}C-atom of glycine (Fig. S3).

Based on our labeling data (Fig. 3) and analyses of mutant strains carrying amino acid exchanges in HypX (Tables S1 and S2 and Figs. S4 and S5), we conclude the following mechanism for the HypX function (Fig. 4). The model involves a two-step reaction mechanism catalyzed by the two HypX modules. In analogy to the catalytic activity of the isolated N-terminal domain of the

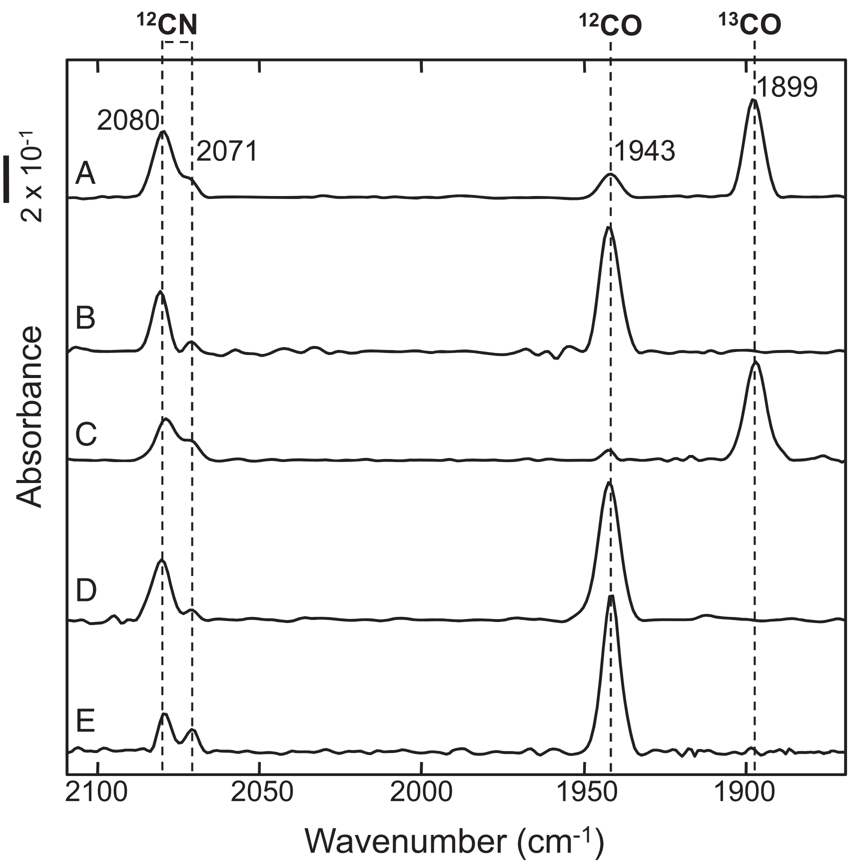

Fig. 3. IR spectra of the RH purified from *glyA*-deficient *R. eutropha* cells (HypX$^+$, Δ*glyA*) and Δ*hypX* mutant cells (HypX$^-$, Δ*glyA*). Cells were cultivated in glycerol medium supplemented with 5 mM of ^{13}C-labeled glycine. (*A*) HypX$^+$ Δ*glyA*, $^{13}C_2$-glycine; (*B*) HypX$^-$ Δ*glyA*, $^{13}C_2$-glycine; (*C*) HypX$^+$ Δ*glyA*, 2-^{13}C-glycine; (*D*) HypX$^-$ Δ*glyA*, 2-^{13}C-glycine; (*E*) HypX$^+$ Δ*glyA*, 1-^{13}C-glycine. Cells were collected at an optical density (436 nm) of ∼1, and RH was purified by affinity chromatography. RH purification from HypX$^-$ strains yielded ∼25% of the wild-type amount. The RH protein was concentrated and subjected to IR spectroscopic investigations. Displayed is the spectral range between 2,120 and 1,870 cm^{-1}. The incorporation of ^{13}CO into the hydrogenase leads to a characteristic shift of the CO-related band to a lower wave number (1,899 cm^{-1}).

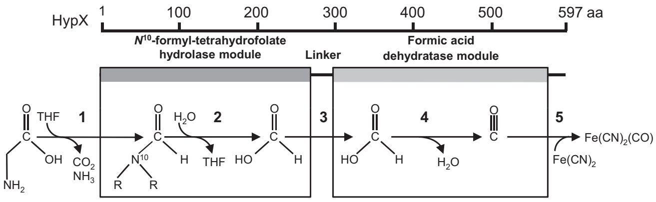

Fig. 4. Model of HypX-mediated biosynthesis of the carbonyl ligand. Cleavage of glycine leads to the formation of 5,10-methylene-THF, CO_2, and NH_3. The intermediate 5,10-methylene-THF becomes oxidized to N^{10}-formyl-THF within the central one-carbon metabolism (R = THF) (1); hydrolysis of N^{10}-formyl-THF within the N-terminal HypX domain and formation of the intermediate formic acid (2); transfer of formic acid to the active site of the C-terminal module (3); dehydration of formic acid to water and CO (4); incorporation of carbonyl ligand into the active site of [NiFe]-hydrogenases (5) (see Fig. S6 for details of the proposed mechanism).

10-formyltetrahydrofolate dehydrogenase (19–22), the N-terminal module of HypX is proposed to act as N^{10}-formyl-tetrahydrofolate hydrolase that converts the formyl group of N^{10}-CHO-THF into the intermediate formic acid (Fig. S6). Subsequently, the C-terminal module of HypX hydrolyses the formic acid into CO and H_2O. This requires the transient stabilization of formic acid ($pK_a = 3.8$ in aqueous solution), which becomes rapidly deprotonated at physiologically relevant pH. In N^{10}-CHO-THF hydrolases, the formic acid intermediate is proposed to be stabilized through hydrogen bond interactions with aspartate and histidine residues (22). Indeed, the corresponding residues D109 and His74 are conserved and functionally important in HypX (23) (Fig. S7). A further protective mechanism to prevent unwanted diffusion and deprotonation of formic acid before reaching the catalytic center in the C-terminal HypX module might be a tunnel-like structure. Tunnels that channel labile reaction intermediates in large enzymes containing multiple catalytic centers are well known (24). Very recently, a formate/formic acid-transporting tunnel has been found to be present in formyl-methanofuran dehydrogenase (25).

The C-terminal part of HypX shares similarity with enoyl-CoA hydratases/isomerases of the crotonase superfamily (13), which catalyze diverse reactions including (de)hydration, isomerization, (de)carboxylation, hydrolysis, and C–C bond formation, all of which involve oxyanion chemistry (26, 27). Two residues, putatively forming the oxyanion hole, are present in HypX (I363 and G416; Fig. S7). Furthermore, the C-terminal module of HypX contains the highly conserved residues Y439, S448, and W451, which are considered as catalytic triad and are also present in nitrile hydratases (Fig. S7). Nitrile hydratases catalyze the hydration of the triple bond-containing nitriles to their corresponding amides (28, 29). In analogy, HypX is proposed to catalyze the dehydration of formic acid, thereby forming water and triple bond-containing CO (Fig. 4 and Fig. S6). This leads to the interpretation that the putative catalytic triad is essential for HypX activity (Fig. S4). Nitrile hydratases use either cobalt or iron for catalysis (28, 29). Interestingly, a maltose binding protein (MBP)–HypX fusion protein heterologously produced in *Escherichia coli* contained 0.9 Fe per protein molecule as determined by inductively coupled plasma optical emission spectrometry (ICP-OES). This is evidence that HypX might contain a metal cofactor.

Although hyperthermal, as well as acid-mediated, dehydration of formic acid into water and CO gas are well-known chemical processes (30, 31), the enzymatic conversion of formic acid into water and CO is unprecedented. As (de)hydratase reactions are principally reversible, the formation of formic acid from H_2O and CO catalyzed by the C-terminal module of HypX is an attractive consideration also under applied perspectives. Toxic CO, a frequent byproduct of chemical reactions, can be used for synthesis of formic acid as a building block in chemical syntheses or as a substrate for direct formic acid fuel cells (32, 33).

Our results show that under physiological conditions at high O_2 partial pressure (low cell density), the availability of CO is a limiting factor in the maturation of the $NiFe(CN)_2(CO)$ cofactor. Therefore, adaption of [NiFe]-hydrogenases to aerobic environments requires dedicated structural adaptions of the enzyme (18) as well as O_2-tolerant cofactor synthesis (34). *R. eutropha* circumvents CO limitation under aerobic conditions by using the auxiliary protein HypX that uses N^{10}-CHO-THF as precursor for CO ligand synthesis. This reaction does not appear to be restricted to *R. eutropha*, as *hypX* is also found in other microbes that use H_2 at ambient O_2 (Figs. S7 and S8). Thus, *hypX* is a crucial factor for those hydrogenases considered to be the major players in the global hydrogen cycle (35, 36). Nonetheless, the anaerobic biosynthesis route of the CO ligand of [NiFe]-hydrogenases awaits to be elucidated.

From an evolutionary point of view, it is interesting to note that the genomes of lower hydrogenase-free eukaryotes such as *Trichoplax adhaerens*, *Puccinia graminis*, and several plants encode HypX-like proteins (Fig. S8). So far, HypX orthologs have been identified only in those eukaryotic genera lacking conventional formyl-THF-dehydrogenases, CO_2-releasing enzymes that are important for recycling oxidized THF derivatives (19). Although enzymatic studies are not yet available, it is attractive to hypothesize that HypX orthologs represent an evolutionary ancestor of formyl-THF-dehydrogenase that has evolved in higher eukaryotes due to the toxicity of CO.

Materials and Methods

For examination of the CO dependence of a *R. eutropha* Δ*hypX* strain, recombinant cells were grown in minimal medium (11) under an atmosphere comprising 15% O_2, 10% H_2, 10% CO_2, and 65% N_2 (all gas concentrations are given in vol/vol). A volume of 0.5% CO was added at the expense of N_2 when indicated. Growth was monitored by measuring the absorption at a wavelength of 436 nm. For isotopic labeling experiments, *R. eutropha* mutant strains were grown in glycerol minimal medium (11) under air in the presence of ^{13}C-labeled glycine derivatives to a final absorption of ∼1.0 (436 nm). The RH protein was purified by affinity chromatography as described previously (11). The isolated protein was applied to IR spectroscopic analysis (7). Details on the experimental procedures can be found in *SI Materials and Methods*.

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