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PROGRESS REPORT FOR DOE Grant No. DE-FG02-87ER13691

TITLE: Carbon Monoxide Metabolism by the Photosynthetic Bacterium *Rhodospirillum rubrum*.

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In the past year, progress was made in: 1) the identification and isolation of the physiological electron carrier from carbon monoxide dehydrogenase (CODH) to hydrogenase in *R. rubrum*; 2) the isolation, sequencing and mutagenesis of the genes encoding the components of the CO oxidation system in *R. rubrum*, 3) the purification and characterization of the CO-induced hydrogenase activity of *R. rubrum*; 4) the spectroscopic investigation of the cobalt-substituted form of the enzyme.

1. Isolation and identification of the physiological electron acceptor between CODH and hydrogenase. While experimenting with variations on the purification protocol for CODH, we noted that CODH could be removed from the membranes of *R. rubrum* by detergents and solvents as an alternative to the heating step employed in our original purification protocol. Deoxycholate was found to release CODH from the membranes far more effectively in the presence of reducing agents than under oxidized conditions. Alternatively, ethanol was found to release the enzyme under either oxidizing or reducing conditions. Purification of either the deoxycholate- or ethanol-solubilized enzyme yielded a form of the enzyme that migrated more slowly during preparative gel electrophoresis than the heat-solublized form. This form had been noted earlier by us as a minor form of CODH. This slower-migrating form was found to have associated with it a 22 kd iron-sulfur protein that serves as both the membrane anchor and the electron acceptor for CODH. It had not been detected earlier because it does not stain well by the silver stain method for proteins; the 22 kd protein stains reasonably well with the coomassie blue protein stain.

Acetonitrile was found to be an effective agent for the separation of the 22 kd protein from the CODH protein (62kd). The ethanol-solubilized CODH (62 kd + 22 kd proteins) were bound to a

DEAE cellulose column. When 30% acetonitrile was present in elution buffer, the 22 kd protein dissociated from CODH and eluted as a tight, brown band. CODH remained bound to the DEAE cellulose column and was eluted subsequently with a higher salt concentration in the absence of acetonitrile. The 22 kd protein was found to specifically allow rebinding of the 62 kd CODH to the membranes and to mediate electron flow from CODH to the membrane bound, CO-induced hydrogenase from *R. rubrum*. Once the gene for the 22 kd protein was identified and mutagenized, the requirement for the 22 kd protein in vivo was established as well (see below). The physical and spectroscopic properties of this protein have been established.

The work described above has been published (J. Biol. Chem. **266**:18395-18403, 1991; a copy of this manuscript is attached)

2. Isolation, sequencing and mutagenesis of the genes encoding the CO oxidation system of *R. rubrum*.

Cloning the *coo* region: We identified a 3.5-kb *Hind*III fragment that hybridized to several degenerate oligonucleotide probes based on the sequence of the purified CODH protein. We have subsequently used this region to probe a lambda library of *R. rubrum* DNA and have subcloned approximately 5 kb on either side of the above *Hind*III region.

Sequencing the cloned region: We have used nested deletions from each end of the cloned region to sequence both strands of the *Hind*III region as well as another 200 bp on one end. Computer analysis, our knowledge of various portions of the sequenced gene product, and mutational analysis (see below) allowed us to identify the reading frame encoding CODH (termed *cooS*, for CO Oxidation, Structural). All or parts of three other ORFs were also noted, as shown in the figure. One of these had a predicted N-terminus that matched the sequenced end of the 22kd protein described above, and was termed *cooF* (*coo* ferredoxin). About 500 bp of the 3' end of another gene was found that had sequence similarity to some hydrogenases (discussed below). This, coupled with mutational data, suggested that it encoded the CO-dependent hydrogenase and the gene was therefore designated *cooH*. Finally, the 5' end of another gene was detected at the opposite end of the clone and showed surprising similarity to *nifH*, but in the absence of other information will be referred to as ORF4.

Mutagenesis of the cloned region: To verify the functionality of the cloned region, and to gain some insight into the biological role of this enzyme system in the organism, we generated polar insertions at several points in the cloned region (see figure), and introduced these into the chromosome of *R. rubrum*. Introductions involved mating the mutated region into *R. rubrum* from *E. coli* on a plasmid incapable of replication in the former strain, selecting for inheritance of the insertion (a kanamycin-resistance cassette), and scoring for the failure to inherit another drug-resistance marker on the vector portion of the plasmid. The resultant strains therefore arose by reciprocal recombination with the chromosome, replacing the wild-type allele with that of the mutant, a fact that was confirmed in each case by Southern analysis. As shown, four mutants were generated, two in *cooS*, and each in *cooF* and *cooH*.

Phenotypic analysis of the constructed mutants: The mutants were examined with a number of different assays that we have developed. These included a CO-dependent, methyl viologen (MV) reduction assay on cells either on plates or in liquid culture, and a direct assay for CO-dependent H_2 production by GC. All four tested mutants were completely defective in the latter assay, which requires that the entire physiological reductant chain be intact.

The two insertions in *cooS* were also completely negative in the MV-based assays, consistent with the model that the gene in question actually encoded the only functional CODH in the cell under these conditions.

The insertion in *cooF* displayed low (4% of wild type) activity in a quantitative MV assay, indicating that it had very low levels of accumulated CODH activity. Given the position and orientation of the *cooF* and *cooS* genes, this would be consistent with polarity of the *cooF* insertion onto *cooS* (the cassette used was not an omega cassette, so some transcription out of the element is possible). It would also be consistent with a requirement for the *cooF* product for the stability of CODH, but this has not been tested. A non-polar insertion in the *cooF* gene is currently being constructed to test this latter possibility.

The mutant with an insertion in *cooH* displayed slightly elevated (138%) levels of CODH activity in the MV assay. This result,

coupled with the sizable space between *cooH* and *cooF*, is consistent with the model that these two regions are separately transcribed. As noted above, the lack of CO-dependent H_2 production in this mutant indicates that the affected gene product is relevant to the CODH system.

Preliminary analysis on the growth of these mutants has begun, with two generalizations possible. Under anaerobic conditions, in the absence of CO, all of the mutants display a wild-type phenotype. In the presence of CO, wild type actually grows at a slightly faster doubling time, suggestive of its use of the CODH system as a "reductant sink". The mutants all grow more slowly in the presence of CO and, with the possible exception of the *cooH* insertion, stop growing at a rather lower cell concentration than does wild type. Variations on these growth experiments are continuing, both to establish the physiological role of the system and to identify growth conditions where selections can be done for a Coo^+ phenotype.

Implications of the gene sequences: All sequenced regions have been analyzed by standard software and databases. The predicted *cooS* product displays considerable similarity to the α subunit of carbon monoxide dehydrogenase of *M. soehngenii* and to the β subunit of the same enzyme from *C. thermoaceticum*. Only the 188 amino acids at the C-terminus of the predicted *cooH* gene product are now known, but this region is similar to the C-terminus of other hydrogenases, particularly the hydrogenase of the formate hydrogenlyase system of *E. coli*, encoded by *hycE*. The predicted *cooF* product is similar to a family of "large" ferredoxins, including the products of *hycB* and *dmsB* of *E. coli*. This family is distinguished by the size (about 20kd) and the presence of four 4-cysteine motifs, several of which are atypical for Fe-S binding regions, but are highly conserved in this gene family.

3. Purification of the CO-induced hydrogenase activity of *R. rubrum*. In order to study a reconstituted system of CO-dependent H_2 evolution in *R. rubrum*, we have initiated the purification and characterization of the CO-induced hydrogenase. This enzyme has proven more refractory to solubilization that other hydrogenases but we have achieved solubilization with triton X-100 and further purification on Q-sepharose.

The solubilized enzyme exhibits very little activity unless membrane fragments are added to the assay mixture suggesting that the hydrogenase activity requires a hydrophobic environment. The activation of solubilized hydrogenase can be achieved with membranes from *R. rubrum* cells that have not been induced for the CO oxidation system and thus the activation appears to be non-specific. As discussed above, it appears that the gene encoding the hydrogenase has been isolated.

The purification and characterization of the hydrogenase has been a goal of this project all along but more emphasis is being placed on this goal in the coming year.

4. Spectroscopic analysis of the cobalt-substituted CODH. Apo-CODH (CODH lacking nickel; purified 62 kd protein from nickel-starved cells) was purified and activated with cobalt. Previous studies in our labs have shown that the cobalt form of the enzyme exhibits very low activity. The cobalt-CODH has been analyzed by UV-visible and EPR spectroscopy. The UV-visible spectra of the enzyme in the oxidized and reduced forms are identical to those of the apo and holo (containing Ni) enzyme. The EPR spectrum of the reduced enzyme is identical to that of the apo and holo enzyme. The EPR spectrum of the oxidized cobalt-CODH consists of a complex signal with multiple features. While we do not yet understand this spectrum, it should provide a useful approach to analysis of the active site of the enzyme.

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