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Progress Report for DE-FG02-87ER13691 (PW Ludden & GP Roberts)
Covering the period 3/15/90 - present.

At the time this funding period was initiated, we had purified and characterized the holo and Ni-deficient forms of carbon monoxide dehydrogenase (CODH) from *Rhodospirillum rubrum* (1, 2), developed protocols for insertion of various metals into the Ni site of the enzyme (3) and characterized these metal substituted forms kinetically and, to a lesser degree, spectroscopically (2, 4, 5). Several inhibitors of CODH had been studied and employed to demonstrate that Ni was at the active site of the enzyme (6, 7). The kinetics and redox requirements for Ni-dependent activation of the Ni-deficient CODH had been described (3). A variety of molecular biology tools had been developed for *R. rubrum* and were ready for application to the CO oxidation system of *R. rubrum*.

In the current grant period, the following advances have been made:

1) Through collaborations with several laboratories, a working hypothesis for the structure of the NiFeS center at the active site of CODH has been developed. One previously attractive model, in which Ni would occupy a postion at the corner of a $NiFe_3S_4$ cubane, has been definitively eliminated. (Tan, Ensign et al. Proc. Natl. Acad. Sci. USA **89**:4427-4431, 1992, copy attached in appendix)

2) A 22 kD FeS protein, which serves as the direct electron acceptor from CODH, has been identified and purified. This Fe_4S_4 protein is required for anchoring CODH to the chromatophore membranes of *R. rubrum* and it is specifically required for reconstitution of CO-dependent H_2 evolution in vitro. (Ensign and Ludden, J. Biol. Chem. **266**:18395-18403, 1991, copy attached in appendix)

3) The *R. rubrum* genes for CODH, the 22 kD (ferredoxin-like) FeS protein, and the CO-induced hydrogenase have been isolated, sequenced, and mutagenized (only part of the hydrogenase gene sequence is completed). This region has been designated the *coo region* (for CO oxidation) with *cooS* encoding CODH, *cooF* encoding the 22 kD ferredoxin and *cooH* the CO-induced hydrogenase. An ORF immediately downstream of *cooS* has been designated *cooC*. The *cooS* and *cooF* genes are cotranscribed, while *cooH* is on a separate transcript (Kerby et al., J. Bacteriol. in press, copy attached in appendix).

4) Insertion mutations have been introduced into the chromosomal copies of *cooS*, *cooF*, *cooH* and *cooC*. The resulting strains have been analyzed for CODH activity as well as for the other functions necessary for CO-dependent H_2 evaluation (Kerby et al., ibid.).

5) The CO-dependent growth of *R. rubrum* has been established in the dark on medium containing only salts plus 0.2% yeast extract under an anaerobic CO gas phase. Under these conditions, *R. rubrum* grows with a doubling time of 5 hours, using CO as the sole energy source and the primary carbon source.

6) The CO-induced hydrogenase activity from *R. rubrum* has been solubilized and partially purified. This hydrogenase is immunologically distinct from other hydrogenases. An in vitro system comprised of CODH, the 22 kD ferredoxin, hydrogenase and undefined factors has been reconstituted to give CO-dependent H_2 evolution.

7) In collaboration with Feinberg's lab, direct electrochemistry has been performed on CODH. (Smith, Ensign, Ludden, Feinberg, submitted to Biochem J.). The

results of these studies confirm our conclusion drawn from dye-mediated redox titrations that the midpoint potentials of the Fe_4S_4 clusters of CODH are -465 mV at pH 7.0 (Ensign et al., Biochemistry **29**:2162-2168, 1990).

These areas of investigation are now described in more detail below.

Spectroscopic studies on the NiFeS cluster of *R. rubrum* CODH. From previous work with the enzyme, a metal composition of $NiFe_8S_{8-10}$ had been determined for the enzyme. EPR spectra of the reduced holo-enzyme or the reduced Ni-deficient enzyme were identical and were best interpreted as corresponding to a pair of Fe_4S_4 clusters. Analysis of the oxidized form of the holo enzyme revealed an EPR signal at g = 2.034 and 1.732 that was broadened by ^{61}Ni and ^{57}Fe, indicating a Ni- and Fe-containing cluster. If the deduced metal composition of the enzyme and the interpretation of the EPR spectra of the reduced enzyme are correct, then the Ni atom of the enzyme must be electonically linked to the FeS clusters in order for the g = 2.034 signal to be broadened by isotopes of both Ni and Fe.

In the current grant period, spectroscopic and biochemical studies of the CODH protein from *R. rubrum* have lead to the working model for the NiFeS cluster of the enzyme presented in Fig 1. EXAFS studies have been employed extensively to define the ligation sphere around the Ni site of CODH and to compare the EXAFS spectrum of *R. rubrum* CODH to the spectra obtained using synthetic compounds of known composition and structure. An extensive set of spectra were collected by our collaborator, R. Korzun, to define the ligation sphere of Ni in the enzyme. In these studies, both oxidized and reduced samples were analyzed at the Brookhaven EXAFS facility. The results are interpreted as >3 sulfur ligands (sulfide, S-cysteine or S-methionine) and one low molecular weight (O or N) ligand. It is interesting that the Ni-S bond lengths of the reduced enzyme are slightly longer than those of the oxidized enzyme. This observation is consistent with the observation that the oxidized, Ni-deficient enzyme cannot be activated by Ni^{2+}, while the reduced, Ni-deficient enzyme can be readily activated. No signal clearly attributable to a Ni-Fe interaction has been observed, thus the Ni-Fe distance must be >3 Å.

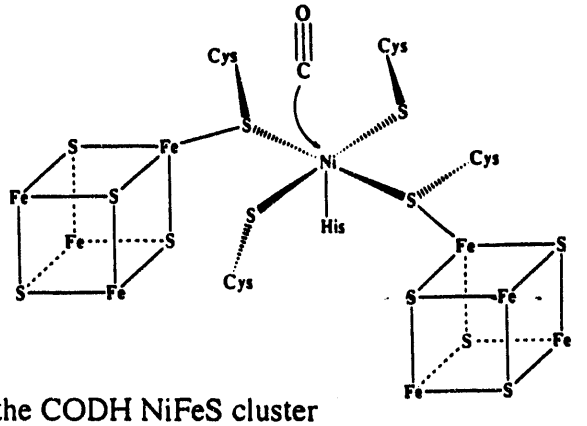

Fig 1. Working Model for the structure of the CODH NiFeS cluster

In a separate set of experiments, Hodgson and co-workers compared the EXAFS spectra of *R. rubrum* CODH to those of $(Et_4N)_3\{NiFe_3S_4(SEt)_4\}$ prepared by Holm and co-workers. The data for the *R. rubrum* CODH from these experiments are in close agreement with the data set collected by Korzun. Furthermore, both data sets are distinctly different from that collected for the $NiFe_3S_4$ cubane in which a strong signal interpreted as a Ni-Fe interaction at a distance of 2.69 Å is observed. These data also confirm our unpublished work with Philip Stephens in which we were unable to observe

any EPR or circular dichroism signal expected of an Fe_3S_4 or Fe_2S_2 center in the Ni-deficient form of the enzyme. If activation of the Ni-deficient form required insertion of Ni^{2+} into the corner of a cubane, an Fe_3S_4-type center would be expected in the Ni-deficient enzyme. Thus, by several independent criteria, *R. rubrum* CODH does not contain a $NiFe_3S_4$ cubane cluster.

At the present time, L edge EXAFS spectra of our enzyme are being collected by Steve Cramer in an effort to define the redox state of the Ni in the oxidized and reduced forms of the enzyme.

In light of our discovery of the organic acid, homocitrate, in the iron-molybdenum cofactor of nitrogenase, we tested for the presence of an organic acid in CODH. No organic acid was detected in the *R. rubrum* CODH.

Identification of the in vivo electron acceptor from CODH. Our published protocol for the purification of CODH employs a heat treatment to release CODH from the chromatophore membranes of the cell extract. When an alternative to this procedure was employed, in which CODH was released by detergent or solvent treatment of the membranes, a lower molecular weight protein co-purified with CODH. It was found that this 22 kD protein could be separated from CODH (with retention of CODH activity) on a DEAE cellulose column. The 22 kD protein was separated from CODH and removed from the DEAE column when the column was washed with buffer containing 50 mM NaCl and 30% acetonitrile.

The 22 kD protein is brown and exhibits UV-visible and EPR spectra characteristic of Fe_4S_4 proteins. The protein contains 4 moles of Fe per mole of protein and is proposed to contain a single Fe_4S_4 cluster. (Note, the presence of four Fe_4S_4 cluster-binding motifs in this protein has been deduced from the gene sequence, see the attached manuscript by Kerby et al.; the significance of the "extra" binding sites is not known.) The protein is oxygen- and heat-labile; the latter explains the ability of the published protocol to yield CODH without associated 22kD protein.

Interestingly, we had previously noted a small amount of a brown band with CODH activity that migrated more slowly than the major band during preparative gel electrophoresis (1, 8). We now know that this is a small fraction of CODH + 22 kD ferredoxin complex that survived the heat treatment. The 22 kD ferredoxin was not detected on SDS gels of this fraction because we always used the silver staining technique and the 22 kD protein stains very poorly by this technique. It is,however, readily observed by coomassie blue staining. Although we refer to the 22 kD protein as a ferredoxin, it would also be reasonable to refer to it as an FeS-containing subunit of the enzyme.

The 22 kD protein re-associates in a 1:1 molar ratio with heat-purified CODH and is proposed to be the in vivo electron acceptor from CODH. CODH that has been purified by either the heat treatment or the detergent/acetonitrile method will reconstitute the 1:1 complex with the 22 kD ferredoxin, thus the heat treatment affects the ability of the 22 kD ferredoxin to bind to CODH but not any observable property of CODH. CODH will mediate the reduction of the FeS center of the 22 kD ferredoxin by CO; the purified 22 kD ferredoxin cannot be reduced by CO in the absence of CODH and it exhibits no CODH activity. The 22 kD ferredoxin also allows re-attachment of CODH to the chromatophore membranes and reconstitution of the CO-dependent H_2 evolution. No other electron carrier tested will substitute effectively for the 22 kD ferredoxin in either respect in our in vitro studies.

Our current scheme for electron flow in the *R. rubrum* CO oxidation system is shown in Fig 2.

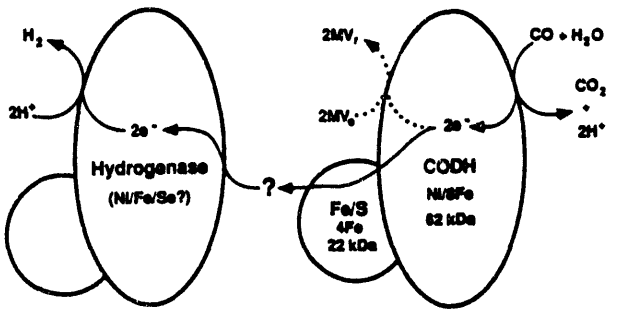

Fig. 2. Proposed Scheme for Electron Flow in the *R. rubrum* CO Oxidation System.

Isolation, sequencing and mutagenesis of the genes encoding the CO oxidation system from *R. rubrum*. The amino acid sequences of cyanogen bromide-generated fragments of CODH were used to design probes for the gene encoding CODH. As described in the attached manuscript by Kerby et al., a 3.7-kb DNA fragment of the *R. rubrum* genome was isolated based on its stong hybridization to one of these probes. The DNA sequence of a portion of this fragment matched the sequence predicted from other, sequenced portions of CODH. Eventually, further sequencing and mutagenesis of this gene demonstrated conclusively that this gene encodes the CODH protein. As this portion of the work was being done, the 22 kD ferredoxin was discovered and purified. The amino acid sequence of the amino terminus of the 22 kD ferredoxin was determined and used to design a probe. This probe also hybridized with the 3.7-kb region and subsequent work demonstrated that the 22 kD ferredoxin was encoded by a gene immediately upstream of the gene encoding CODH. These genes have been given the designations *cooS* (for CODH) and *cooF* (for the ferredoxin). When the region upstream of *cooF* was sequenced, strong similarity to known hydrogenase genes was noted and, based on subsequent biochemical analysis of strains with mutations in this gene, it has been designated *cooH*. A region downstream of *cooS* has been designated *cooC*. Although the role of the product of *cooC* in CO oxidation is not yet known, insertions in this gene reduce CODH activity 50-fold, suggesting a role in CODH processing, function or stability. The arrangement of genes in the *coo* region is shown in Fig 3. The DNA sequence and the inferred amino acid sequences of the *coo* region are shown in Fig. 3 of the attached manuscript by Kerby, et al.

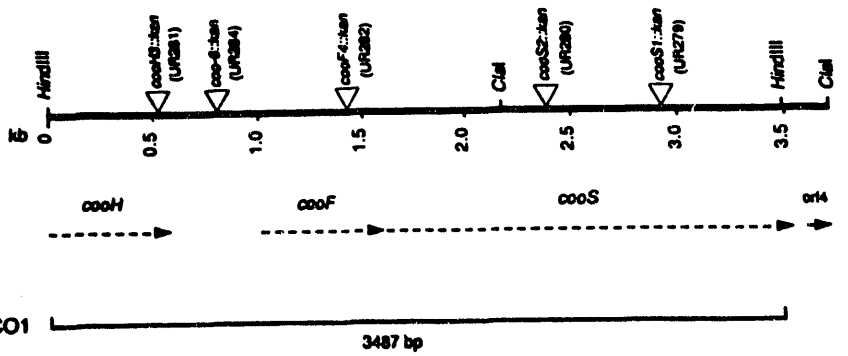

Fig. 3. The *coo* gene cluster in *R. rubrum*.

Analysis of the CODH sequence reveals that it is most similar to the β subunit of the *Clostridium thermoaceticum* CODH (67% sequence similarity) and the α subunit of the *Methanothrix soenghenii* CODH (47%) (9, 10). Our results with the monomeric *R. rubrum* CODH, in which there is no ambiguity as to which subunit may hold the Ni site, should provide insight into the Ni sites of the more complex enzymes from *C. thermoaceticum* and *M. soenhghenii*. There are numerous cysteines in the protein sequence but no obvious Fe_4S_4 cluster-binding motif. A region of CODH (A_{120}HSEHGRHIAL in *R. rubrum*)is highly conserved in CODHs from all three organisms. This might be a Ni or FeS cluster-binding site or a site involved in processing Ni to its final site in the enzyme; the EXAFS data described above are not consistent with this sequence serving as the Ni-binding site in the mature protein.

As noted above, the *cooF* sequence suggests multiple sites for Fe_4S_4 cluster binding, but only a single cluster is observed in the isolated protein. X-ray crystallography studies proposed here will address the number and arrangement of FeS clusters in the enzyme. The sequence of the 22 kD ferredoxin shows similarity to a number of FeS proteins involved in energy conservation. These similarities and the similarity of gene arrangement among a number of systems are described in the attached manuscript by Kerby et al. from our laboratories.

Kanamycin-resistance cassettes were used to disrupt each of the genes described above and the mutated genes were reintroduced into the *R. rubrum* genome by homologous recombination. Biochemical and genetic analyses of the mutant strains show that *cooF* is polar onto *cooS*, but *cooH* is on a separate transcript. The $cooH^-$ mutant lacks CO-dependent hydrogenase activity in vivo and in vitro but exhibits normal levels of CODH activity in extracts, strongly suggesting that *cooH* indeed encodes the CO-induced hydrogenase activity. It is interesting that the $cooH^-$ mutant exhibits normal levels of pyruvate-dependent H_2 evolution when cells are grown under dark, anaerobic conditions, indicating that a distinct gene(s) encodes the hydrogenase responsible for the pyruvate-dependent activity.

CO-dependent growth of *R. rubrum*. Uffen had previously reported growth of *R. rubrum* on CO (11, 12), and we wished to characterize this further. A series of experiments to determine the growth characteristics of *R. rubrum* in the presence of CO under a variety of conditions (aerobic, anaerobic, dark and light conditions) was conducted. From these experiments, we have learned that *R. rubrum* will grow with a doubling time of 5 hours on a medium containing only salts and 0.2% yeast extract under an atmosphere of CO. Dark anaerobic growth is completely dependent on the presence of CO and the growth yield depends on the initial concentration of CO in the gas phase. Mutant strain UR279 (*cooS::kan* ; lacks CODH activity) is unable to grow on the salts + yeast extract + CO medium. CO-dependent growth is readily observable on plates and can be used to select against mutants defective in the energy conservation or CO-autotrophic components of the CO oxidation system of *R. rubrum*.

Purification of the CO-induced hydrogenase. Characterization of this hydrogenase and description of its special properties (including the relative insensitivity to CO, compared to other hydrogenases) are essential to understanding the CO oxidation system of *R. rubrum*. Though this protein has been difficult to purify, in part because it is so tightly membrane bound, substantial progress has been made in purification in the past year. Membranes containing the enzyme are washed with CHAPS buffer which removes many proteins (including CODH and the 22 kD ferredoxin) but not the

hydrogenase. Hydrogenase is then solubilized by Triton X-100 which is then removed before loading the enzyme on a DEAE cellulose column. Hydrogenase runs through this column, but many proteins, and especially the pigment proteins, remain bound. Column fractions with hydrogenase activity are then loaded onto a Q-Sepharose column and hydrogenase activity is eluted with a salt gradient. The Q-Sepharose column effects an 8-fold purification of the enzyme. The active fractions from the Q-Sepharose column are brown in color, probably because of the presence of hydrogenase, which we expect to be an NiFeS protein.

Cells grown anaerobically in the dark with CO as the carbon and energy source have 2- to 3-fold higher levels of both hydrogenase and CODH activities than cells grown phototrophically with malate and CO as carbon sources. Because many of the experiments proposed in the Experimental section require large amounts of enzymes, CO-grown cells will be used as the source of enzyme for purification in future studies.

A tetrazolium dye reduction assay has been adapted for localizing the hydrogenase activity on native acrylamide gels of fractions and has allowed identification of the hydrogenase protein band on native gels (13). In future studies, we should be able to extract that band and electrophorese the proteins on denaturing SDS gels and identify the hydrogenase spot(s) on these gels.

Antibodies obtained from the Arp lab (14) have been used to test for immunological cross-reactivity of the CO-induced hydrogenase with other hydrogenases. No cross-reactivity was observed using antibody against the purified uptake hydrogenase from *Azotobacter vinelandii*; this antibody shows broad reactivity with Ni-hydrogenases and thus the lack of reactivity of the *R. rubrum* CO-induced hydrogenase with this antibody suggests that it does not fall in this class of enzymes even though physiological evidence suggests Ni is required for hydrogenase activity.

Efforts to crystallize CODH. The crystallization and determination of the crystal structures of CODH and the 22kD ferredoxin will be major goals of the coming grant period. We have initiated attempts to crystallize CODH in collaboration with Dr. Hazel Holden of the Enzyme Institute on the Madison campus. Crystals have been obtained, but none of sufficient quality to begin data collection.

(References cited in the Progress Report are listed in the Reference Section following the proposal)

List of publications from this grant period:

Tan, G.O., S.A. Ensign, S. Ciurli, M.J. Scott, B. Hedman, R.H. Holm, P.W. Ludden, Z.R. Korszun, P.J. Stephens and K.O. Hodgson. 1992. On the structure of the nickel/iron/sulfur center of the carbon monoxide dehydrogenase from *Rhodospirillum rubrum*: An X-ray absorption spectroscopy study. Proc. Natl. Acad. Sci USA, 89: 4427-4431.

Ensign, S.A. and P.W. Ludden. 1991. Characterization of the CO oxidation/H_2 evolution system of *Rhodospirillum rubrum:* role of a 22-kDa iron-sulfur protein in mediating electron transfer between carbon monoxide dehydrogenase and hydrogenase. J. Biol. Chem., 266: 18395-18403.

Ensign, S.A., M.J. Campbell and P.W. Ludden. 1990. Activation of the nickel-deficient carbon monoxide dehydrogenase from *Rhodospirillum rubrum*: Kinetic characterization and reductant requirement. Biochemistry, 29: 2162-2168.

Kerby, R.L., S.S. Hong, S.A. Ensign, L.J. Coppoc, P. W. Ludden and G.P. Roberts. Genetic and physiological characterization of the *Rhodospirillum rubrum* carbon monoxide dehydrogenase system. J. Bacteriol. In Press.

Smith, E.T., S.A. Ensign, P.W. Ludden and B.A. Feinberg. Direct electrochemical studies of hydrogenase and CO dehydrogenase. Submitted to Biochem. J.

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