1. PROGRESS REPORT on DE-FGO2-88ER13875

Mechanism of formation of the carboxyl of acetate by acetogenic bacteria

1. Summary of accomplishments during the second year of support

The overall goal of this project is to understand how the carboxyl of acetate is formed by acetogenic bacteria. Our work involves a multidisciplinary approach towards understanding the details of this process which is a key step in the pathway by which anaerobic bacteria synthesize acetyl-CoA. We have used a number of techniques including spectroscopy, enzyme kinetics, electrochemistry, and molecular biology. Figure 1 summarizes our current concept for how this pathway occurs. Our work focuses on carbon monoxide dehydrogenase (CODH).

During this year, the sequences of the genes encoding the two subunits of CODH have been determined in a collaboration between the labs of Lars Ljungdahl (Univ. of Georgia) and myself (Morton et al, in preparation). Thus, we now know the complete amino acid sequence of CODH.

We made major progress in studies of the structure and function of metal centers in the nickel, iron-sulfur protein, CODH, by Mössbauer (1) electron nuclear double resonance (ENDOR) (Fan et al, in preparation), and EPR (2) spectroscopic techniques and coulometric (2) studies. CODH was found to contain one [4Fe-4S] cluster, a [Ni-Fe-C] center, and a [2Fe] center with two oxidation reduction potentials (1, 2). In a description of the Mössbauer work we postulate that the site for binding CO is a mixed metal center that contains a [4Fe-4S] center bonded to a nickel site by a ligand bridge (1).

One of the metal centers on CODH is the methyl binding site (3). We discovered that methylation of CODH by the methylated-C/Fe-SP involves reductive activation of a metal center followed by formation of the methyl-metal intermediate (3).

1.1. Determination of the DNA sequence of the genes encoding key proteins involved in acetyl-CoA synthesis

We isolated, cloned into *Escherichia coli*, and mapped five separate genes from *Clostridium thermoaceticum* which encode key proteins (CO dehydrogenase, CODH; the corrinoid/iron-sulfur protein, C/Fe-SP; and methyltransferase, MeTr) in this pathway (Fig. 1, bottom). These genes constitute a ~ 11 kb gene cluster and are expressed at levels in *E. coli* as high as 10% of cell protein (4). These proteins encode the steps in this pathway which are unique features of the Wood pathway which is the major mechanism of CO_2 fixation under anaerobic conditions. Genes encoding these or analogous proteins in other anaerobes have not been cloned or sequenced from any anaerobe, thus we are in a position to provide the first primary structures of these proteins as well as to make seminal contributions to the study of the regulation of the pathway. We found that the gene encoding the large subunit of CODH is directly downstream

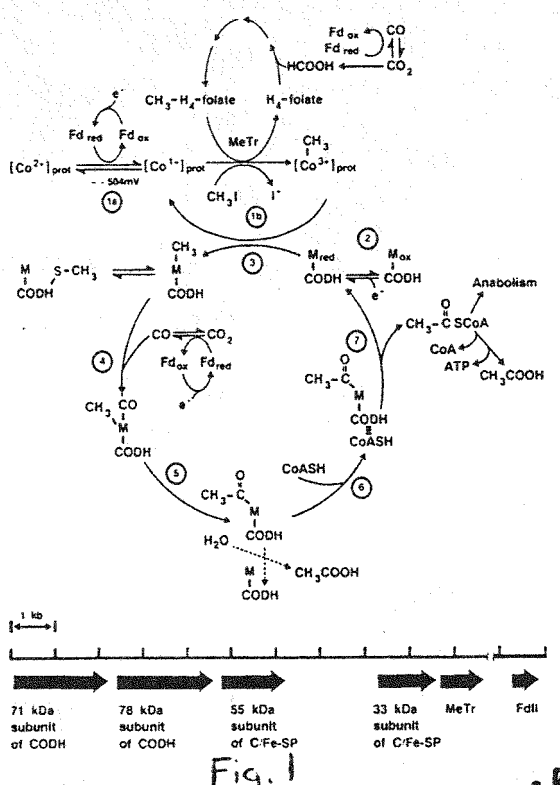

Fig. 1

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Office of Basic Energy Sciences, HQ
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SUBJECT: GRANT NO. DE-FG02-88ER13875 - UNIVERSITY OF WISCONSIN

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of the gene encoding the small subunit. In this past year, in a collaboration with Lars Ljungdahl (U. of Georgia) the complete amino acid sequences of the two subunits of CODH were determined (Fig. 2) by sequencing the genes encoding these proteins. Since this is the first amino acid sequence of a CODH yet reported we cannot use sequence homology to ensure that some mistake in reading the DNA sequence did not occur. The most common mistake in reading DNA sequences is a frameshift error caused by insertion or deletion of a nucleotide. This would be a serious error since, from the beginning of the frameshift until the end of the protein, the predicted sequence could be entirely wrong. In order to ensure that the sequence was aligned properly, we determined the sequence of random tryptic peptides isolated from CODH by trypsin digestion of the native protein and located three of these peptide sequences in the predicted primary sequence. We also compared the frequency of codon usage in CODH to another sequenced *C. thermoaceticum* gene, methylene-H_4folate reductase, and found the predicted codon usage to be highly probable. This codon usage method was used recently to correct the predicted amino acid sequence of a hydrogenase (5). The incorrect deletion or addition of a base is detected by a radical shift of the probable codon usage frequency to another frame. In addition, there are only minor differences between the predicted and the experimentally determined amino acid compositions. The predicted molecular weights of the two subunits are 73 kDa and 82 kDa and the molecular weights determined by SDS electrophoresis are 71 kDa and 78 kDa; thus these values are quite similar. Based on these combined criteria, we are confident that the sequence shown in Fig. 2 is correct.

SMALL SUBUNIT OF CODH

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M P R F R D L S H N C R P S E A P R V M E P K N R D R T V D P A V L
E M L V K S K D D K V I T A F D R F V A Q Q P Q C K I G Y E G I C C
R F C M A G P C R I K A T D G P G S R G I C G A S A W T I V A R N V
G L M I L T G A A A H C E H G N H I A H A L V E M A E G K A P D Y S
V K D E A K L K E V C R R V G I E V E G K S V L E L A Q E V G E K A
L E D F R R L K G E G E A T W L M T T I N E G R K E K F R T H N V V
P F G I H A S I S E L V N Q A H M G M D N D P V N L V F S A I R V A
L A D Y T G E H I A T D F S D I L F G T P Q P V V S E A N M G V L D
P D Q V N F V L H G H N P L L S E I I V Q A A R E M E G E A K A A G
A K G I N L V G I C C T G N E V L M R Q G I P L V T S F A S Q E L A
I C T G A I D A M C V D V Q C I M P S I S A V A E C Y H T R I I T T
A D N A K I P G A Y H I D Y Q T A T A I E S A K T A I R M A I E A F
K E R K E S N R P V Y I P Q I K N R V V A G W S L E A L T K L L A T
Q N A Q N P I R V L N Q A I L D G E L A G V A L I C G C N N L K G F
Q D N S H L T V M K E L L K N N V F V V A T G C S A Q A A G K L G L
L D P A N V E T Y C G D G L K G F L K R L G E G A N I E I G L P P V
F H M G S C V D N S R A V D L L M A M A N D L G V D T P K V P F V A
S A P E A M S G K A A A I G T W W V S L G V P T H V G T M P P V E G
S D L I Y S I L T Q I A S D V Y G G Y F I F E M D P Q V A A R K I L
D A L E Y R T W K L G V H K E V A E R Y E T K L C Q G Y *
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LARGE SUBUNIT OF CODH

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M T D F D K I F E G A I P E G K E P V A L F R E V Y H G A I T A T S
Y A E I L L L N Q A I R T Y G P D H P V G Y P D T A Y Y L P V I R C F
S G E E V K K L G D L P P I L N R N V A Q V S P V L N F E N A R L A
G E A T W Y A A E I I E A L R Y L K Y K P D E P L L P P P W T G F I
G D P V V R R F G I K M V D W T I P G E A I I L G R A K D S K A L A
K I V K E L M G M G F M L F I C D E A V E Q L L E E N V K L G I D Y
I A Y P L G N F T Q I V H A A N Y A L R A G M M F G G V T P G A R E
E Q R D Y Q R R R I R A F V L Y L G E H D M V K T A A A F G A I F T
G F P V I T D Q P L P E D K Q I P D W F F S V E D Y D K I V Q I A M
E T R G I K L T K I K L D L P I N F G P A F E G E S I R K G D M Y V
E M G G N R T P A F E L V R T V S E S E I T D G K I E V I G P D I D
Q I P E G S K L P L G I L V D I Y G R K M Q A D F E G V L E R R I H
D F I N Y G E G L W H T G Q R N I N W L R V S K D A V A K G F R F K
N Y G E I L V A K M K E E F P A I V D R V Q V T I F T D E A K V K E
Y M E V A R E K Y K E R D D R M R G L T D E T V D T F Y S C V L C Q
S F A P N H V C I V T P E R V G L C G A V S W L D A K A S Y E I N H
A G P N Q P I P K E G E I D P I K G I W K S V N D Y L Y T A S N R N
L E Q V C L Y T L M E N P M T S C G C F E A I M A I L P E C N G I M
I T T R D H A G M T P S G M T F S T L A G M I G G G T Q T P G F M G
I G R T Y I V S K K F I S A D G G I A R I V W M P K S L K D F L H D
E F V R R S V E E G L G E D F I D K I A D E T I G T T V D E I L P Y
L E E K G H P A L T M D P I M *
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Fig. 2

1.2. Determination of the structures and roles of the metal centers in CODH

We also made major progress in identifying the metal centers in CO dehydrogenase (CODH). This protein has the remarkable property of reducing carbon dioxide to carbon monoxide at redox potentials very near the thermodynamic potential for this reaction and also to allow the bacterium to use carbon monoxide, a toxic gas, as a source of carbon and energy. These interesting properties of the protein are due to some special metal centers in this enzyme which are extremely complicated. CODH contains 2 Ni, 12 Fe, and 14 $S^{=}$. We have used Mössbauer, ENDOR, and EPR spectroscopic techniques as well as spectroelectrochemical methods to identify and determine the the midpoint redox potentials of the metal centers in CODH. Two manuscripts have been accepted in the J. Biol. Chem. based on the EPR and Mössbauer work

(1, 2) and we are preparing a manuscript describing some of the ENDOR work.

1.2.1. Characterization of the unique metal center (Ni-Fe-C center) responsible for binding of CO and other C-1 donors

Determining the structure of the site of binding of CO to CODH is extremely important since it is a key intermediate step in the pathway of acetyl-CoA synthesis (Fig. 1). It also is the site of activation of CO or CO_2 activation on the enzyme. When CODH is incubated with acetyl-CoA under reducing conditions, an EPR signal is formed which is identical to the signal formed upon incubation of CODH with CO. This EPR signal had been shown previously to originate from a metal center containing Ni, Fe, and the C of CO (termed the Ni-Fe-C center) (6). Discovering that this Ni-Fe-C signal was produced from acetyl-CoA is extremely interesting for two reasons: first, it directly implicates this center in not only binding CO and also in the binding of the carbonyl of acetyl-CoA, and, second, it allowed us to determine the midpoint redox potential of the Ni-Fe-C center.

In a collaboration with Eckard Münck and Paul Lindahl (a postdoctor for Münck at the time), we studied this Ni-Fe-C center by EPR, Mössbauer, and controlled potential coulometry. It was not possible to determine accurately the redox potential for the Ni-Fe-C center with these experiments, but based on this work we are able to make a reasonable postulate concerning the structure of the site on CODH for binding CO (1, 2). CODH was labeled with ^{57}Fe by growing the bacteria in media containing this isotope and the Mössbauer spectrum of this center is shown in Fig. 3C. This spectrum arises from a center containing 3-4 irons, based on the absorbance of this feature relative to the total iron absorbance in the protein. Three to four irons also was predicted from simulations of the EPR spectra (6, 7). The spectrum in 3C was obtained by subtracting the spectrum in Fig. 3B from that in Fig. 3A. The sample in 3A had 0.35 spins/mol and in 3B contained only 0.1 spin/mol of Ni-Fe-C EPR signal. The solid line is a spectral simulation with an S = 1/2 spin Hamiltonian assuming two sites with the same quadrupole splitting and isomer shift values, ΔE_Q = 1.15 mm/s, δ = 0.44 mm/s, but distinct magnetic hyperfine tensors; $A_x = A_y$ = 33.5 MHz and A_z = 29.5 MHz for site 1 and A_x = 33.5 MHz and $A_y = A_z$ = 25 MHz for site 2. Further evidence that this spectrum of Fig. 3C represents the spectral contribution of the Ni-Fe-C complex was obtained by performing Mössbauer at high temperatures. We knew, based on EPR studies, that at temperatures above 60 K, the spin of the Ni-Fe-C complex relaxes slowly on the Mössbauer time scale, whereas, the spins of the other Fe-S-containing centers relax fast. Thus, the spectra of the iron sites of the Ni-Fe-C complex will exhibit magnetic hyperfine structure and all other paramagnetic centres will contribute quadrupole doublets. Based on these parameters, we predict that the iron atoms are in the form of a [4Fe-4S] center. The values listed above which were used in this spectrum of Fig. 3C were successful in simulating the magnetic Mössbauer spectrum at 90 K. The ΔE_Q and δ values are surprising since they predict that the overall oxidation state of the cluster is the 2+ state, yet this center is paramagnetic. A standard [4Fe-4S] ferredoxin-like center is paramagnetic in the 1+ state and diamagnetic in the 2+ state. We postulate, based on

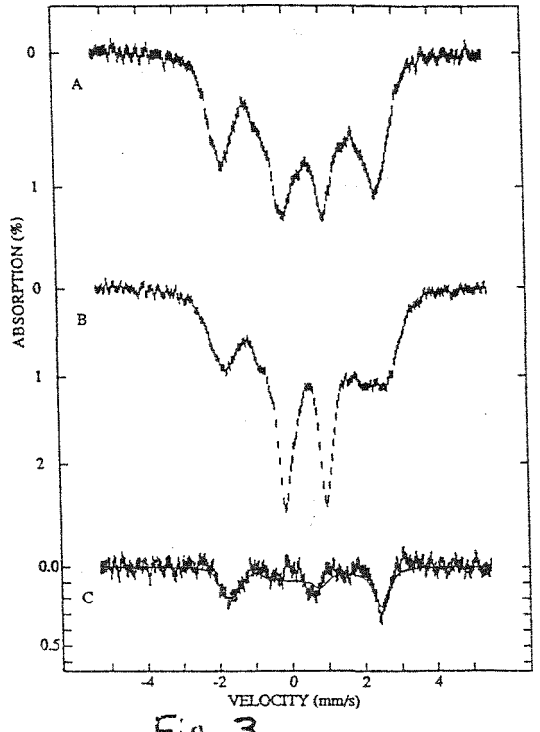

Fig. 3

this data, that the Ni-Fe-C center contains a [4Fe-4S] center bonded to a nickel site by a ligand bridge (1, 2). In this model, we predict that the formal oxidation state of Ni is 1+ which is paramagnetic and that of the cluster is 2+. This would give rise to a paramagnetic S = 1/2 system.

The above interpretation is consistent with recent studies by ENDOR spectroscopy in a collaboration with Brian Hoffman (Northwestern Univ.). We have detected ^{61}Ni, ^{57}Fe, and ^{13}C ENDOR signals from CO-reacted CODH (Fig. 4). The magnetic hyperfine splitting values are: A^{Ni}, 24.3 MHz; A^{Fe1}, 27.4 MHz and A^{Fe2}, 34.9 MHz; and A^{C}, 28 MHz (isotropic). The iron hyperfine splitting values are similar to those used to simulate the Mössbauer data. The ENDOR data provide unambiguous evidence that when CODH is reacted with CO, a novel metal center is formed which is composed of iron, nickel and the carbon of CO. This also is the first reported ENDOR signal from a nickel-containing enzyme.

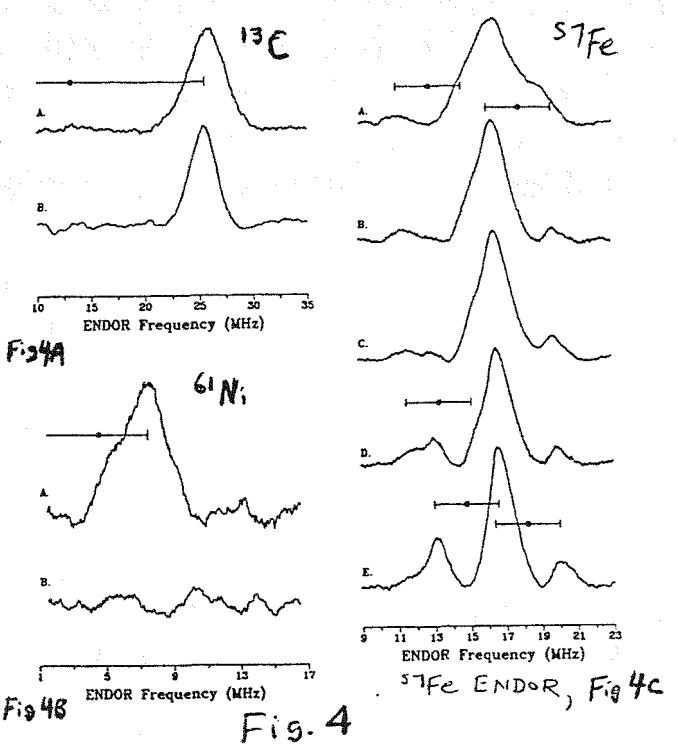

Fig. 4

Recently, we have been successful in determining the midpoint redox potential of the Ni-X-[4Fe-4S]-CO center by incubation of CODH with acetyl-CoA at negative redox potentials. We were surprised initially to discover that under these conditions, a stable complex is formed in which the Ni-Fe-C signal develops Previous attempts to do this had failed since the only known method for generating this EPR signal is by treatment with CO or CO_2 under reducing conditions. When CO is reacted with the enzyme, the redox potential of the solution plummets to ~-650 mV and, when the enzyme is electrochemically reduced in the presence of CO_2, CO is produced and the redox potential of the solution is dependent upon the CO/CO_2 redox couple. Thus, there is little control over the redox potential of the solution in the presence of these substrates. Though acetyl-CoA also is a substrate of CODH, it does not itself undergo redox transformations and one can titrate the EPR signal as a function of potential (Fig. 5). The Nernst plot of the data (inset, Fig. 5) gives a midpoint redox potential of -530 mV.

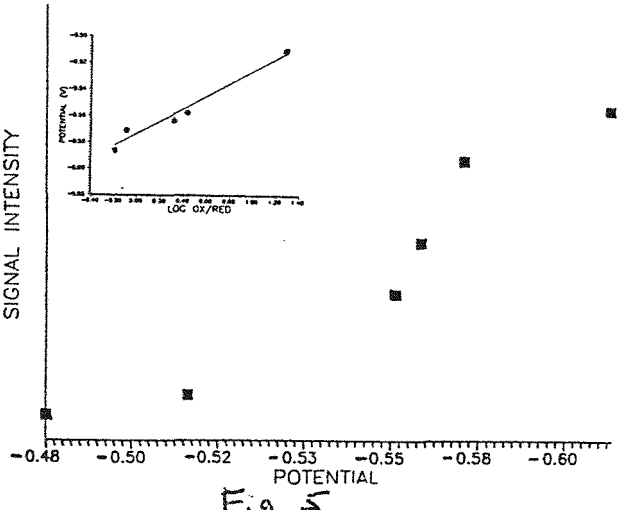

Fig 5

1.2.2. Characterization of the other metal centers in CODH

Besides the Ni-Fe-C center, we have attempted to fully characterize the metal centers in CODH. EPR and controlled potential coulometric studies coupled with Mössbauer studies have identified one [4Fe-4S] cluster that can exist in two conformations, both with g values at 2.04, 1.94, 1.90, but with different linewidths. The two conformers have E_o's of -390 and -490 mV, the

broader one having the more negative potential. There is a center that apparently has 2 Fe and possibly Ni and can exist in two EPR-active conformations with different g-values, one at 2.01, 1.76, 1.65 (g_{ave} = 1.82) and one at 1.97, 1.86, 1.75 (g_{ave} = 1.86). Therefore, there are three oxidation states, the g_{ave} = 1.82 species with E_o' of -215 mV and the g_{ave} = 1.82 species with E_o' of -530 mV. The description of this work is in press at the J. of Biological Chemistry (1, 2).

In our preliminary studies, the midpoint potentials for these metal centers has a fairly high uncertainty (± 40 mV) which was possibly due to the design of the electrochemical cell. A new cell has been designed (8) which has recently resulted in very accurate and repeatable data for the titration of the metal centers in CODH. The new values are similar to those reported above but the error is ± 10 mV. In addition, we had noticed quite a bit of variability in the EPR spectra from one sample to another. With the new design, the titrations are much more reproduceable.

We are interested in determining the roles of the other metal centers in CODH. We discovered that one of the metal centers in CODH is the site of methylation by the methylated C/Fe-SP (see Fig. 1). This is a key step in the pathway since CODH transforms this active methyl group into the methyl of acetyl-CoA. We have discovered that methylation of CODH requires a reductive activation of a metal center on CODH at redox potentials more negative than -400 mV. We discovered this by studying the methylation of CODH with the methylated C/Fe-SP and also two exchange reactions: one between methyl-CODH and the methyl of acetyl-CoA (Equation 1) and one between methyl-CODH and the methyl-C/Fe-SP (Equation 2). We found that the

$$^{14}CH_3\text{-CODH} + CH_3\text{-CO-SCoA} \quad CH_3\text{-CODH} + {}^{14}CH_3\text{-CO-SCoA} \quad (1)$$

$$^{14}CH_3\text{-CODH} + CH_3\text{-C/Fe-SP} \quad CH_3\text{-CODH} + {}^{14}CH_3\text{-C/Fe-SP} \quad (2)$$

exchange between methyl-CODH and the methyl of acetyl-CoA is ~100x faster at low redox potentials than at ambient potentials (~0 mV). The kinetic competence of this methylated CODH intermediate was established by studying these exchange reactions and by performing the synthesis of acetyl-CoA from CH_3-CODH, CO, and CoA. One mol of methyl was bound to CODH and in the exchange reaction with the methylated CODH and the methyl of acetyl-CoA, 95% of this methyl was exchanged. In addition, ~80% of the methyl was converted to acetyl-CoA. The results of these experiments were submitted for publication to the Journal of Biological Chemistry. More recent evidence concerning the identification of this methyl binding site is being sought by ENDOR spectroscopy of the reduced $^{13}CH_3$-CODH. Preliminary results indicate that a methyl binding site is on a [4Fe-4S] cluster.

1.3. Characterization of an exchange reaction between the carboxyl of pyruvate and the carbonyl of acetyl-CoA

We are interested in how the carboxyl of pyruvate is converted into the carbonyl of acetyl-CoA and are attempting to identify intermediates formed during this process by spectroscopy. We have studied this conversion by NMR methods (Fig. 6) by following an exchange reaction between the carboxyl of pyruvate and the carbonyl of acetyl-CoA (Equation 3). When carboxyl-labeled pyruvate is

$$CH_3CO^{13}COOH + CH_3COSCoA \quad CH_3COCOOH + CH_3{}^{13}COSCoA \quad (3)$$

incubated with CODH in the presence of pyruvate-ferredoxin oxidoreductase (PFOR), we find that there is a time- and enzyme-dependent exchange reaction in which the ^{13}C label is found in the carbonyl of acetyl-CoA. Other studies (Gorst and Ragsdale, unpublished) have shown that the exchange between the carbonyl of acetyl-CoA and CO proceeds through the Ni-Fe-C intermediate

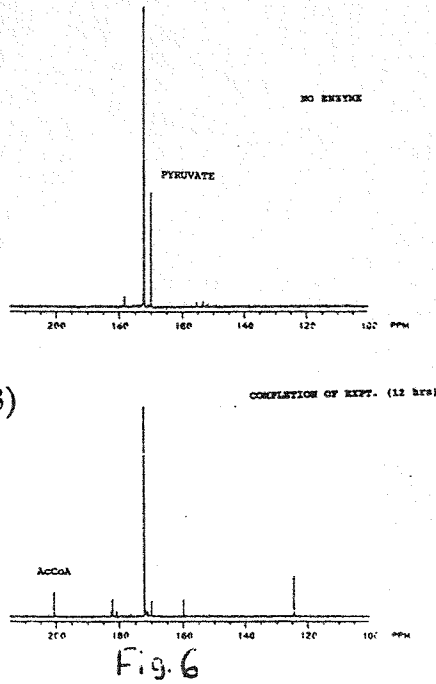

Fig. 6

described above. Thus, this reaction also implies that the Ni-Fe-C intermediate is involved in pyruvate metabolism. The simplest explanation for how this reaction would occur is a direct transfer of the carboxyl of pyruvate to the spin-coupled Ni-X-[4Fe-4S] center on CODH. Further experiments to clarify the pathway of this reaction are discussed below.

PROPOSAL FOR THE UPCOMING YEAR

Prospects for the next year:

Our progress during the past year has helped us understand some key details in the process of acetyl-CoA synthesis and also forms the background for our further studies. There are very few changes in the direction of the project. In the next year we plan to:

1. Perform structural studies on CODH, attempting to identify the ligands to the metal centers and the substrate binding sites.
2. Further characterize the metal centers in CODH.
3. Determine the bond order of the CO complex bound in the Ni-Fe-C center.
4. Study the effect of carbonyl sulfide on the reaction mechanism.
5. Characterize the exchange reaction between the carboxyl of pyruvate and the carbonyl of acetyl-CoA.

1. Structural studies on CODH

We hope in the upcoming year to begin to identify the ligands to the metals in CODH and locate the CoA, methyl, and CO binding sites in the primary sequence. We have determined the DNA sequence of CODH from *C. thermoaceticum*. Now our goal is to relate this sequence to structural features in the protein. CODH catalyzes extremely interesting chemical reactions. We would like to know the locations and orientations of the metal centers and the location of the CO, CoA, and methyl binding sites on CODH. In the absence of a three dimensional structure, we will use more indirect methods to locate these sites.

1.1. Localization of the ligands to the metal centers in CODH

In the identification of the ligands to the metals in the Ni-Fe-C center which is responsible for binding CO, we will use several methods. This work complements the work described below on the spectroscopic determination of the structures of the metal centers in CODH.

1.1.1. Protease treatment

We will attempt to isolate a proteolytic fragment of CODH which retains metal binding. We know (Ragsdale, unpublished) that Ni is very tightly bound to CODH and that Ni is involved in binding CO. We hope that by digestion with trypsin and other proteases, we can excise this CO binding domain. For these experiments, we will isolate CODH from cells grown in the presence of ^{63}Ni and treat with protease for varying periods of time. These samples will be electrophoresed under non-denaturing conditions. Enough protein will be loaded that the brown color due to Fe-S clusters can be detected and also the gel will be autoradiographed to determine the location of the nickel as a function of time of digestion. Hopefully, a small fragment can be isolated which retains Ni and Fe. If this is successful, we will electroelute the protein onto a glass fiber membrane for direct amino acid sequence analysis. Then we can locate this Ni-binding domain in the primary sequence and thus predict where CO binds to CODH. We were successful in using

this methodology to separate and sequence the N termini of the two subunits of CODH.

In this digestion, Fe-S centers can be located as brown bands on the nondenaturing PAGE and these will be sequenced as well in the manner just described. Thus we hope to also locate the binding sites for the other Fe-S centers in CODH and compare these sequences to the complete sequence of the protein to locate metal binding sites in the primary sequence.

1.1.2. Sequence comparisons

A number of Fe-S containing proteins have been sequenced and many of these contain a cysteinyl block: $C\text{-}X_2\text{-}C\text{-}X_2\text{-}C\text{-}X_n\text{-}C$ (see ref. 13, for example). We will search the amino acid sequence for sequences which are homologous to this sequence. Cysteine blocks which could form Fe-S clusters are circled in Fig. 2. We will compare the secondary structures of these cysteinyl blocks with those of other Fe-S proteins as well.

1.2. Location of the CoA and methyl binding sites in the primary sequence

I have described one method for locating the CO binding site by protease digestion. We have just obtained the amino acid sequence of CODH and are now beginning to relate this structure to that of other proteins. We are attempting to locate the CoA binding site by two methods. A number of CoA binding protein have been sequenced and we are comparing their primary and secondary structures with those of CODH. In addition, the crystal structure of citrate synthase is known (11). It has been speculated that the CoA binding site is similar to that of nucleotide and phosphate binding sites in proteins (12), so we will also compare our primary and secondary structures with that of these regions in other proteins. Harland Wood's laboratory has isolated peptides containing which are protected by CoA against N-bromosuccinimide modification and are in the process of sequencing these peptides. Once these sequences are available, we will be able to locate them in the primary sequence. Wood's group also has found that the methyl group can bind to a cysteinyl residue in CODH. He is attempting to obtain a peptide in which the S-methyl group remains intact and sequence this peptide. We feel that this cysteinyl methyl binding site is quite close to the CO binding site and is close to the metal center which is the active site for methyl binding. So localization of this specific methyl cysteine will aid in the localization of the metal centers involved in CO and methyl binding. Since CO binds to CODH in a labile manner at the Ni-Fe-C site and the methyl group also forms a labile complex at a metal center, it is unlikely that the direct approach with protease treatment and isolation and sequencing of the peptide would be successful.

2. Further characterization of the metal centers in CODH

We have been successful in our preliminary characterization of the metal centers of CODH by EPR, Mossbauer, and controlled coulometric studies (see above for summary). Besides the publishable results, we learned quite a lot about CODH from these studies. We have discovered that CODH, although homogeneous in terms of mobility on polyacrylamide gels and on other criteria for purity, is heterogeneous in terms of its magnetic and electronic properties. There are two forms of almost every metal center: the Ni-Fe-C center has two conformations distinguishable by EPR; the [2Fe] center has two conformations distinguishable by EPR and interconvertable as a function of redox potential; the [4Fe-4S] cluster also has two forms, distinguishable by EPR linewidth, relaxation properties, and each with distinct midpoint redox potentials. At the present time, we have little control over the relative amounts of each conformer and, clearly, a careful study of conditions which interconvert or favor one conformer over the other has to be performed.

In addition, the metal centers quantitate to only ~0.5 spins per mol. We do not as yet know what form or the state of the other 0.5 missing spin.

In one series of studies, we will investigate the cause of these inhomogeneities and low spin quantitations and try to determine which of the conformers are characteristic of active enzyme, or if both are characteristic of active enzyme. This will include careful documentation of relative amounts of each species in each preparation based on simulation of the different features in the EPR spectra and determination of the activity in each reaction: CO oxidation, CO_2 reduction, CO/acetyl-CoA exchange, and acetyl-CoA synthesis.

In ENDOR spectroscopic studies, we will focus on characterizing the other Fe-S centers in the enzyme. A major goal is to identify the metal site which acts as a methyl acceptor on CODH from the methylated C/Fe-SP. In these experiments, which have already begun, we methylate CODH with $^{13}CH_3I$ in the presence of the C/Fe-SP at low redox potentials and immediately transfer the sample to a small quartz EPR tube which is used by Hoffman and his coworkers for Q-band ENDOR measurements.

3. Vibrational spectroscopy of CODH

We have begun to perform FT-IR and Resonance Raman spectroscopic studies of CODH. Our major focus is on detection of the metal carbonyl intermediate formed on CODH on reaction with CO. This information is essential to determine the bond order of the bound CO (a terminal CO or bridging C=O) and how many different types of CO binding sites are on the protein. We are using the CIRCLE cell which is an apparatus which allows infrared spectroscopy to be performed in aqueous solution. Initially, we have detected the Fe-CO bond of hemoglobin which has been reported. This was essential in order to maximize the sensitivity of the instrument. We can now easily detect this vibrational band and have begun to study CODH. Initial attempts with CODH are not encouraging, however, this theoretically is a valuable source of information and we will devote the necessary time and effort to improve conditions until we can detect it. It may be necessary to compare the ^{13}CO- and ^{12}CO-reacted enzymes and detect the bond by difference spectroscopy. Hopefully the Resonance Raman spectroscopic studies will be well along within the year as well.

4. Inhibition with carbonyl sulfide

Hyman et al (9) discovered that the CODH from *Rhodospirillum rubrum* is strongly inhibited by carbonyl sulfide in a manner competitive with CO (K_i = 2.3 μM) and uncompetitive with the electron acceptor, methyl viologen. It was proposed that COS acted as a CO rather than as a CO_2 analogue. We plan to determine the effect of COS on the kinetics of the CO oxidation, CO_2 reduction, CO/acetyl-CoA exchange, and acetyl-CoA synthesis reactions of CODH. We also will determine if COS can act as a substrate. We expect that it will serve as a CO_2 analogue, based on its structure, and will determine if it can act as an electron acceptor in our electrochemical system. If so, it is expected that COS, under reducing conditions, would produce CO and H_2S. We will detect CO production with an assay based on observing the change in the hemoglobin spectra on binding CO (10). We also will determine the effect of COS on the EPR spectra of CODH. If it acts as either a CO or CO_2 analogue, we expect to see the Ni-Fe-C EPR signal. We do not yet know the oxidation state of the C bound to this center or how many oxygens are bound. If either a [formate] or [CO_2] (with two oxygens) are bound to CODH, we would expect to see an EPR spectrum which reflects the bound COS which would be different than our normal EPR spectrum.

5. Characterization of the exchange reaction between the carboxyl of pyruvate and the carbonyl of acetyl-CoA

We will continue the NMR studies on CODH to fully characterize the transfer of the carboxyl of pyruvate from PFOR to the carbonyl of acetyl-CoA. The ^{13}C NMR experiments characterizing the exchange between the labelled carboxyl of pyruvate and the carbonyl of acetyl-CoA (see above, Fig. 6) show several resonances by the end of the experiments. Most of these resonances have been assigned by comparison to published values, however the resonance at 178ppm has not been unambiguously identified. We have tentatively assigned this resonance to CO based on NMR experiments following the exchange between CO and $^{13}C_1$-acetyl-CoA by CODH, however the published resonance position for CO is at 181.3 ppm. In these CO/acetyl-CoA exchange experiments two resonances were observed. The first resonance at 200.4 ppm decreased in intensity throughout the experiment and was assigned to acetyl-CoA (this is the published value for the carbonyl of acetyl-CoA). The second resonance occured at 178.0 ppm and increased in intensity throughout the experiment. Since ^{13}CO is the expected product of the exchange (14), the resonance at 178.0 was assigned as ^{13}CO. Another potential product is acetate; the published resonance position for acetate is 177.3 ppm. Thus, either of these species could account for the resonance at 178 ppm. Based on the results of the CO/acetyl-CoA exchange it was assumed that the peak at 178 ppm in the pyruvate exchange experiments was CO. To identify this resonance unambiguously it will be necessary to repeat the experiments and to spike the reaction mixture with either ^{13}CO or ^{13}C-acetate.

We would also like to determine which of the species seen as a product of the exchange reaction between pyruvate and the carbonyl of acetyl-CoA are involved in the exchange process and which are the result of substrate or product degradation. One technique for doing this is magnetization transfer NMR. Magnetization transfer techniques for the analysis of enzyme-mediated exchange processes are extremely valuable since they can provide the rate constant for the exchange process as well as identify which resonances are involved in the exchange (15).

In addition to the magnetization transfer experiments, we would also like to characterize the EPR signal generated by reacting pyruvate with CODH in the presence of PFOR. Based on the NMR experiments of the exchange reaction of acetyl-CoA with pyruvate, it is expected that the carboxyl of pyruvate can be transferred directly to CODH and is likely to have the ability to generate the Ni-Fe-C signal when incubated with CODH under conditions similar to those in the NMR experiments.

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Budget Justification

This budget is for the third and final year of the present grant and reflects the level of funding committed for the upcoming year. The PI is requesting one-month summer salary. I will perform the FT-IR and Resonance Raman experiments on CODH. The salary for a 100% effort postdoctor is requested for Dr. Jennifer Runquist. Dr. Runquist is a senior-level postdoctor who has been in the lab for ~ 1-1/2 years and has performed the sequencing of the CODH genes. She will be involved in structural studies of CODH, locating the substrate binding sites and the metal binding sites. The salary of a graduate assistant also is requested for Carol Gorst, who is a third year Ph.D. student. Ms. Gorst has performed the EPR, NMR, and enzymological experiments on CODH. In the upcoming year, she will continue her Ph.D. project by performing the protease experiments and the spectroscopic and electrochemical studies to further characterize the structures of the metal centers in CODH. Ms. Gorst also will perform the carbonyl sulfide inhibition experiments and the NMR studies of the exchange reaction between the carboxyl of pyruvate and the carbonyl of acetyl-CoA. These projects all aim toward understanding the mechanism of CO_2 and CO fixation by anaerobic bacteria and how they utilize this pathway of acetyl-CoA synthesis in the generation of energy.

Manuscripts in press since last year on work supported by DOE funding:

1. Roberts, D.L., James-Hagstrom, J.E., Smith, D.K., Gorst, C.M., Runquist, J.A., Baur, J.R., Haase, F.C., and Ragsdale, S.W. (1989) Cloning and expression of the gene cluster encoding key proteins involved in acetyl-CoA synthesis in *Clostridium thermoaceticum*: CO dehydrogenase, the corrinoid/Fe-S protein, and methyltransferase, Proc. Natl. Acad. Sci., **86**, 32-36.
2. Lu, W.-P., Harder, S.R., & Ragsdale, S.W. Methyl transfer reactions involved in acetyl-CoA synthesis, J. Biol. Chem., in press.
3. Lindahl, P.A., Münck, E., and Ragsdale, S.W. CO dehydrogenase from *Clostridium thermoaceticum*: EPR and electrochemical studies in CO_2 and argon atmospheres, J. Biol. Chem., in press.
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Manuscripts submitted or in preparation since last year on work supported by DOE:

1. Fan, C.-L., Gorst, C.M., Ragsdale, S.W., and Hoffman, B.M. Studies of CO dehydrogenase using electron nuclear double resonance, in preparation.
2. Morton, T., Runquist, J.A., Ragsdale, S.W., and Ljungdahl, L.G. Cloning and DNA sequence analysis of the CO dehydrogenase from *Clostridium thermoaceticum*, in preparation.