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Identification of a cysteine involved in the interaction between carbon monoxide dehydrogenase and corrinoid/Fe-S protein from *Clostridium thermoaceticum*

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In *Clostridium thermoaceticum*, the synthesis of acetyl-CoA from methyl tetrahydrofolate occurs via a series of enzymatic reactions involving methyl transferase, corrinoid/Fe-S protein (corrinoid), carbon monoxide dehydrogenase (CODH) and ferredoxin. We have investigated the possibility of one or more of these proteins existing as multi-enzyme complexes in vivo with higher catalytic activity. A protein complex consisting of CODH and corrinoid was isolated from the cell-free extracts of *Clostridium thermoaceticum*. The acetyl-CoA synthesis was found to be ~ 1.8-fold higher with the complex than that observed with the isolated protein components. HPLC gel filtration analyses of the native and DTE reduced complex suggested that the CODH:corrinoid complex is held together primarily by an inter disulfide bond. By differential labeling of thiols with [^{14}C]*N*-ethylmaleimide it was found that Cys-506 of the α subunit of CODH was involved in the disulfide linkage with the corrinoid of the complex.

Carbon monoxide dehydrogenase; Corrinoid; Protein–protein interaction; Acetyl-CoA synthesis; Protein complex; *Clostridium thermoaceticum*

1. INTRODUCTION

The acetogenic bacterium, *Clostridium thermoaceticum*, synthesizes acetyl-CoA by a pathway which involves: (i) the reduction of CO_2 to methyltetrahydrofolate, (ii) the transfer of the methyl group by methyl transferase to CO dehydrogenase (CODH) through a corrinoid, and (iii) subsequent binding of CO and CoA to CODH forming acetyl-CoA [1,2]. Fuchs [3] has estimated that glucose is consumed in *Clostridium thermoaceticum* at the rate of 115 μmol per min per g of cell and suggested that the in vitro rates of activities reported for the enzymes involved in acetyl-CoA synthesis are much lower than that predicted for in vivo rates. Based on this, he proposed that an important link in the synthetic process is missing. Wood and Ljungdahl [2] postulated that the enzymes of the acetyl-CoA pathway may occur as multi-enzyme complex in cellular milieu with activities much greater than that determined in the isolated state.

In the present investigations, we have successfully isolated a complex of CODH and corrinoid and showed that acetyl-CoA synthesis is greater with the protein complex than that obtained with the isolated enzyme components. Also, we have determined the amino acid sequence of CODH comprising the CODH–corrinoid interaction site.

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Abbreviations: CODH, carbon monoxide dehydrogenase; NEM, *N*-ethylmaleimide; TFA, trifluoroacetic acid; Corrinoid, corrinoid/Fe-S protein; HS-CoM, methyl coenzyme M.

2. MATERIALS AND METHODS

2.1. *Chemicals*

N-Ethylmaleimide (NEM) and sequanal grade trypsin were purchased from Sigma. ^{14}C-labeled NEM was from Amersham. Acetonitrile was from EM Sciences and trifluoroacetic acid was obtained from Pierce.

2.2. *Enzymes*

CODH was prepared under anaerobic conditions and assayed as described by Ragsdale and Wood [4]. Methyltransferase and ferredoxin were purified according to the procedures of Drake et al. [5]. Corrinoid protein was isolated by the procedure outlined in [6] and the acetyl-CoA synthesis was monitored as in [5].

2.3. *Purification of CODH:corrinoid complex*

Unless otherwise mentioned, all purification steps were carried out under anaerobic conditions. About 100 g of the wet cells of *Clostridium thermoaceticum* were suspended in 50 mM Tris-HCl buffer, pH 7.6 containing 2 mM DTE (buffer I) and lysed with a French press. The lysate was centrifuged at 27,000 rpm for 1 h. The supernatant was loaded on to a DEAE-Sephacel column. CODH and corrinoid were eluted together at 0.2 M NaCl using a linear gradient of 0–0.5 M NaCl in buffer I. Ammonium sulfate was added to a final concentration of 0.35 M to the fractions containing CODH and corrinoid and applied to a phenyl-Sepharose column previously equilibrated with buffer I containing 0.35 M ammonium sulfate. A linear negative gradient, generated from buffer I containing 0.35 M ammonium sulfate and ammonium sulfate-free buffer I, was used to elute the protein complex. The fractions containing both CODH and corrinoid, as evidenced by SDS-PAGE, were pooled and further purified on a Sephadex G-100 column by eluting with buffer I.

2.4. *Differential labeling of thiols of CODH in the CODH:corrinoid complex*

The CODH:corrinoid complex was subjected to differential labeling of thiols using *N*-ethylmaleimide (NEM) as described in [7,8]. Briefly, non-radiolabeled NEM was added to a final concentration of 5 mM to block the free -SH groups of the CODH:corrinoid complex. The reaction was allowed to continue for 1 h at room temperature. The cold NEM-treated complex was passed through a Bio Gel P6 DG desalting column to remove the excess reagent and the inter disulfide bonds of the complex were reduced by incubating with 20 mM DTE for 1 h. The complex was subsequently passed through a Bio Gel A-1.5 m column which was previously equilibrated with 50 mM Tris-HCl, pH 7.6 containing 20 mM DTE. The fractions containing CODH were identified by SDS-PAGE. Alternatively, the two proteins were also separated using a DEAE-Sephacel column and the proteins were eluted using a linear gradient of 0–0.5 M NaCl. The NEM modified CODH was treated with a solution of [^{14}C]NEM (5 mM; 2000 cpm/nmol) in 0.5 ml of 50 mM Tris-HCl buffer, pH 7.6 and the reaction was allowed to continue for 1 h at room temperature. The reaction mixture was passed through a Bio Gel P6 DG desalting column to remove the excess reagent. In a parallel experiment, CODH alone which served as a control was subjected to differential labeling protocol as described above.

2.5. *HPLC analysis of CODH differentially labeled with [^{14}C]NEM*

The [^{14}C]NEM-labeled CODH was digested with trypsin for 18 h (1:50, trypsin/CODH) and the resulting peptides were separated by HPLC using a C-18 reverse-phase column by monitoring at 220 nm. The mobile phase constituted 0.1% TFA (solvent A) and 0.1% TFA in acetonitrile (solvent B). The fractions were collected and the radioactivity in each fraction was determined by using a Beckman Model LS 9000 counter. The fractions containing the radioactivity were lyophilized and the labeled peptide was re-purified by a second HPLC step which employed a shallow gradient.

2.6. *Amino acid sequence and composition analysis*

The amino acid sequence of the labeled peptide was determined using an Applied Biosystems Model 477A amino acid sequencer equipped with an on line PTH amino acid analyzer (Model 120A). The PTH fractions were collected and the radioactivity was determined as described above. The amino acid composition of the peptides was determined as described in [9].

3. RESULTS

3.1. *Characterization of the CODH:corrinoid complex*

We have isolated a protein complex of CODH and corrinoid from the cell-free extracts of *Clostridium thermoaceticum* using a relatively low concentration of DTE during purification. As evidenced by SDS-PAGE, the isolated CODH:corrinoid complex contains four protein components with molecular weights of 78,000, 71,000, 55,000 and 35,000 (Fig. 1). The protein bands corresponding to 78,000 and 71,000 arise from CODH and those corresponding to 55,000 and 35,000 are from the corrinoid enzyme [4,6]. HPLC gel filtration analysis further indicated that the isolated complex contained both CODH and corrinoid protein in 1:1 stoichiometry (Fig. 2 and Table I).

To determine if the two proteins were held together by a disulfide bond, the CODH:corrinoid complex was treated with 20 mM DTE for 1 h at 18°C and analyzed by HPLC gel filtration. The elution profile of DTE pre-treated complex was compared with the profiles obtained from the untreated complex and the constituting

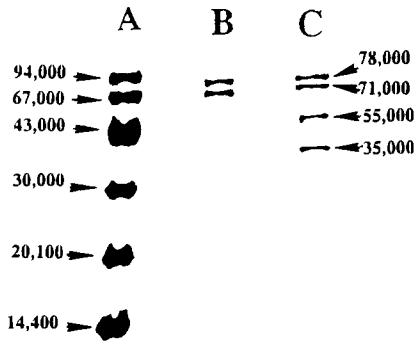

Fig. 1. SDS-PAGE analysis of the CODH:corrinoid complex isolated from *Clostridium thermoaceticum*. Lane A, molecular weight markers consisting of phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and lactalbumin (14,440); lane B, CODH; and lane C, CODH:corrinoid complex.

proteins (Fig. 2). The untreated complex eluted as a single peak (Fig. 2C) which resolved into two protein peaks (peaks 1 and 2) following incubation with DTE (Fig. 2D) and were identified with CODH and corrinoid, respectively (Fig. 2A,B). The results indicated that CODH and corrinoid are linked together by disulfide bonds in the isolated complex.

The complex was assayed for its ability to synthesize acetyl-CoA and compared with the activity obtained using isolated enzyme components. The results of Table I indicate that the synthesis of acetyl-CoA by the complex is ~ 1.8 times more than that synthesized by the individual components. This observation suggests that a close interaction between CODH and corrinoid is essential for an increased synthesis of acetyl-CoA.

3.2. *Amino acid sequence at the contact site of CODH and corrinoid*

In order to identify the thiol(s) of CODH involved in the disulfide bond formation, the complex was subjected to differential labeling with NEM as described in

Table I

Acetyl-CoA synthesis catalyzed by the CODH:corrinoid complex and isolated enzyme components

Sample	Protein content (mg)		Acetyl-CoA synthesis (nmol)
	Corrinoid	CODH	
CODH:corrinoid complex	0.19	0.17	312
CODH+corrinoid	0.18	0.18	172

Protein concentration of the components of the complex isolated by procedures described in section 2 was determined by the Rose Bengal method [20]. The reaction mixture contained either CODH:corrinoid complex (0.36 mg) or isolated CODH (0.18 mg) and corrinoid (0.18 mg). Other components included in the assay for acetyl-CoA synthesis were: ferredoxin (0.1 mg), methyltransferase (0.1 mg), CoA (0.8 mmol) and CH_3THF (700 cpm/nmol; 2 mmol). Average values from four independent experiments are presented.

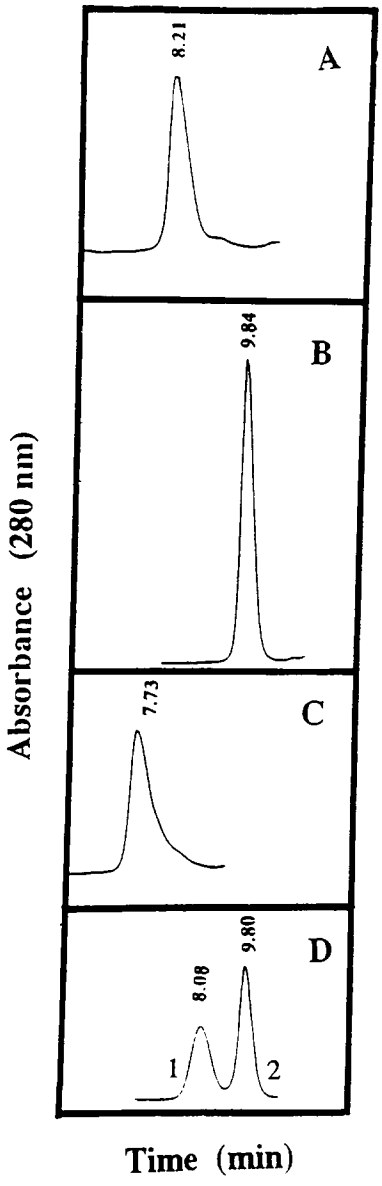

Fig. 2. HPLC gel filtration analysis of CODH:corrinoid complex. CODH or corrinoid or CODH:corrinoid complex was subjected to HPLC gel filtration on a G4000SW column. For protein separation, 100 mM Tris-HCl buffer, pH 7.6 was used and the flow rate was 1 ml per min. Panel A, CODH alone; panel B, corrinoid alone; panel C, CODH:corrinoid complex; and panel D, CODH:corrinoid complex treated with 20 mM DTE for 1 h prior to gel filtration.

section 2. Based on the amount of [^{14}C]NEM incorporated, it was calculated that one nmol of the reagent was bound to 1 nmol of CODH following the dissociation of the complex by DTE. When CODH alone was used in the differential labeling studies the incorporation of [^{14}C]NEM was only at background levels. The radiolabeled CODH was digested with trypsin and the peptides were separated using reverse phase HPLC. The fraction which eluted at 35% acetonitrile concentration (Fig. 3, indicated by asterisk) contained more than 90% of the radioactivity applied. This peptide fraction was further purified by a second HPLC step to homogeneity and subjected to amino acid sequence and composition analyses. As shown in Table II, the amino acid sequence of the radiolabeled peptide corresponds to residues 494–508 of the α subunit of CODH [10]. The radioactivity was eluted in the 13th cycle which corresponds to Cys-506. Amino acid composition analysis and a comparison of the peptide sequence with the primary structure of CODH indicated that the cleavage occurred at Arg-493 and Arg-524 of the α subunit of CODH [10]. Taken together, these observations suggest that Cys-506 of the α subunit of CODH is involved in the disulfide bond formation with a thiol of corrinoid protein.

4. DISCUSSION

In acetogenic and methanogenic organisms, association of enzyme components essential for either synthesis of acetate or for the conversion of acetate to methane has been proposed [3,6,11,12]. Hu et al. [6] have suggested that the corrinoid and CODH from the acetogen, *Clostridium thermoaceticum*, may form a complex in vivo. In *Methanobacterium barkerii*, the efficient transfer of methyl group from methanol to HS-CoM appears to require a multi-enzyme complex [12]. The suggestion that the enzymes of the acetyl-CoA pathway in *Clostridium thermoaceticum* may assemble in vivo into multi-enzyme complexes [2] stems from the need to account for the differences in the rate of acetate synthesis attainable in vitro using isolated components as compared to the rate predicted in vivo during fermentation of glucose [3]. The observation that an electrostatic complex between CODH and ferredoxin could be formed with an associated stimulation of carbonyl exchange activity lends support to this hypothesis [13]. Furthermore, as demonstrated in this study, complex formation via inter disulfide bond does occur in vivo between CODH and corrinoid with facilitatory effects on the overall acetyl-CoA synthesis. Recently, Ragsdale and his co-workers have obtained higher in vitro rates of acetyl-CoA synthesis using a low potential system [14]. It is possible

Table II

Amino acid sequence of the α subunit of CODH at the CODH:corrinoid contact site

	494										506
This study:	Gly-	Leu-	Thr-	Asp-	Glu-	Thr-	Val-	Asp-	Thr-	Phe-	Tyr-Ser- **Cys**-Val-Leu-
From DNA:	Gly-	Leu-	Thr-	Asp-	Glu-	Thr-	Val-	Asp-	Thr-	Phe-	Tyr-Ser- **Cys**-Val-Leu-

The Cys residue at the 13th cycle of sequencing contained the radioactivity.

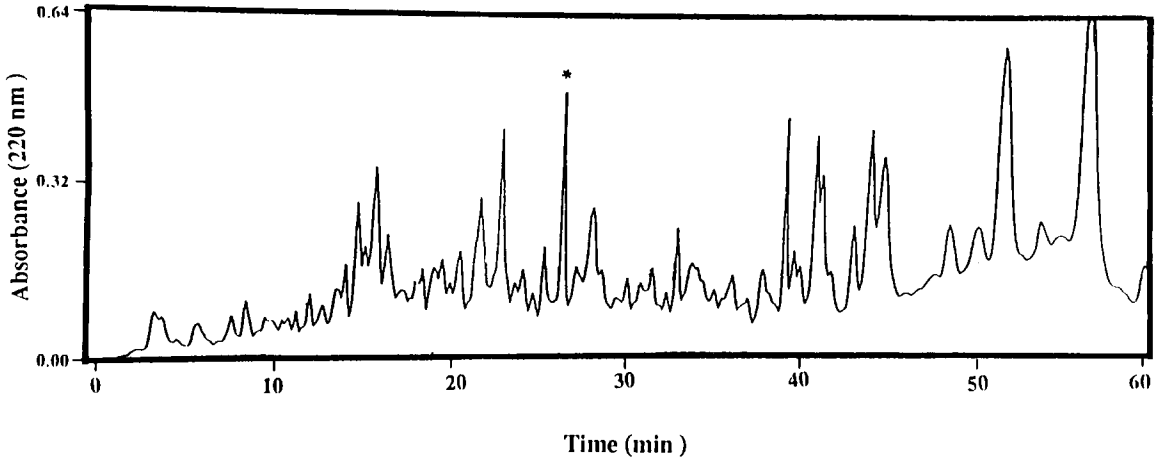

Fig. 3. HPLC peptide profile of CODH differentially labeled with [^{14}C]NEM. CODH differentially labeled with [^{14}C]NEM was digested with trypsin and the tryptic peptides were separated by HPLC. The other experimental details are as described in section 2.

that under low potential system CODH and corrinoid may adopt conformations different from that which prevail during ambient conditions and this could facilitate effective protein–protein interaction with enhanced acetyl-CoA synthesis.

Previously, a complex containing CODH and corrinoid was isolated by Terlesky et al. [15] in *Methanosarcina thermophila*, but no functional studies on the complex were carried out. It has been recently demonstrated that the CODH:corrinoid complex from *Methanosarcina thermophila* synthesizes acetyl-CoA from methyl iodide, CO and CoA [16,17]. Further, Raybuck et al. [18] have shown that a similar complex from *M. thermophila* also catalyzes the acetyl-CoA-CO exchange as does CODH from *Clostridium thermoaceticum*. Our present findings clearly provide evidence for the existence of a functionally active complex of CODH and corrinoid in *Clostridium thermoaceticum* as in certain methanogens.

The involvement of cysteine residues at the contact site enabled us to determine the amino acid sequence of CODH that involves in binding of corrinoid. Ragsdale and his coworkers have proposed that the methyl group from the corrinoid is transferred to a metal center of CODH [19]. It is likely that the methyl binding site is located in the vicinity of Cys-506 of the α subunit of CODH. The presence of potential metal binding amino acids such as Asp, Glu, Cys and Gly in the sequence of CODH at the corrinoid contact site (Table II, [10]) lends support to this possibility. Electron microscopic studies of CODH:corrinoid complex is currently underway in our laboratory to understand the topography of the interaction site.

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