Decomposition of Carbon Monoxide Dehydrogenase into α Metallosubunits and a Catalytically-Active Form Consisting Primarily of β Metallosubunits†

Jinqiang Xia and Paul A. Lindahl*

Department of Chemistry, Texas A & M University, College Station, Texas 77843

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ABSTRACT: Carbon monoxide dehydrogenase from *Clostridium thermoaceticum*, with an $\alpha_3\beta_3$ quaternary structure, was incubated with dithiothreitol and 740−78 000 equiv/$\alpha_3\beta_3$ of sodium dodecyl sulfate (SDS), followed by electrophoresis under anaerobic native conditions. Three catalytically-active bands and four inactive bands were obtained, in proportions dependent on the amount of SDS added. The catalytically-active bands, called SM-CODH, NM-CODH, and FM-CODH, migrated slower than, similar to, and faster than native enzyme, respectively. Two-dimensional electrophoresis revealed that SM-CODH and NM-CODH contained approximately equal proportions of the α and β subunits, while the β/α ratio for FM-CODH was about 2.7. The four inactive bands were identified as the β subunit, two forms of the α subunit (called α and α′), and a form that migrated similarly to native enzyme. Overloaded gels revealed that α and each active band had brown color, indicating intact Fe-S clusters in these species. Higher concentrations of SDS (>1600 equiv/$\alpha_3\beta_3$) and/or incubation at temperatures >15 °C yielded more α and β subunits at the expense of the catalytically-active bands. When incubated at 70 °C in 78 000 equiv/$\alpha_3\beta_3$ of SDS, α quantitatively converted to α′, suggesting that α′ is a denatured form. FM-CODH appears to be an intermediate in the decomposition of CODH by SDS and to have either an $\alpha_1\beta_3$ or $\alpha_1\beta_2$ quaternary structure. FM-CODH catalyzed CO oxidation at essentially the same turnover frequency as NM-CODH, indicating that the dissociable subunits are not involved in the oxidation of CO. The β subunits appear to contain the clusters required for CO oxidation, while α subunits may function in the synthesis of acetyl-CoA.

Carbon monoxide dehydrogenase from *Clostridium thermoaceticum* (CODH[1]) catalyzes the reversible oxidation of CO to CO_2 and, in conjunction with three other proteins (the corrinoid/iron−sulfur enzyme, methyltransferase, and a ferredoxin), the synthesis of acetyl-CoA (Ragsdale, 1991; Wood & Ljungdahl, 1991). The substrates for the synthase reaction, namely CO, CoA, and the methyl group from methyltetrahydrofolate, bind to sites on CODH.

CODH has an $\alpha_3\beta_3$ quaternary protein structure (Ragsdale et al., 1983), with molecular mass 464 000 Da. The α and β subunits have masses of 81 730 and 72 928 Da, respectively (Morton et al., 1991). Each $\alpha\beta$ unit contains 2 Ni and 11−13 Fe, organized into at least four types of centers (Lindahl et al., 1990a,b) including: (i) the A-cluster,[2] (ii) the C-cluster, (iii) Fe_4S_4 clusters, and (iv) ferrous component II. The two types of catalytic reactions almost certainly occur at different active sites. The A-cluster is probably the active site for acetyl-CoA synthesis (Lu & Ragsdale, 1991; Gorst & Ragsdale, 1991; Shin & Lindahl, 1992a,b; Shin et al., 1993; Kumar et al., 1994), while the C-cluster appears to be the active site for CO oxidation (Anderson et al., 1993; Kumar et al., 1994). The Fe_4S_4 clusters transfer electrons between these active-site centers and external redox agents (Kumar et al., 1994; Anderson & Lindahl, 1994), while the function of ferrous component II is unknown. With the exception of the Fe_4S_4 clusters, the structures of these metal centers are unknown and probably unique.

The A-cluster appears to consist of a Ni complex chemically linked to a large Fe-S cluster. When reduced by one electron and bound with the substrate CO, the complex is $S = {}^1/_2$ and yields the so-called NiFeC EPR signal. The intensity of this signal is unusually low, quantifying to a maximum of only *ca.* 0.3 spin/$\alpha\beta$. The Ni in this complex can be removed and reinserted quantitatively (Shin & Lindahl, 1992a,b), but only ~1 Ni/$\alpha_3\beta_3$ can be removed/replaced (Shin et al., 1993). When considered along with the low spin intensity of the NiFeC signal, Shin et al. suggested that each $\alpha_3\beta_3$ molecule may contain only one A-cluster. This raises the possibility that the enzyme consists of a single functioning $\alpha_3\beta_3$ entity with various unique clusters, rather than three identical independently-functioning $\alpha\beta$ units with identical copies of clusters. This possibility diminishes the prospects of determining the exact number and structures of metal centers in the enzyme, for it essentially triples the complexity of an already-complicated metalloenzyme.

We have endeavored to simplify this problem by developing a means to separate the α and β subunits without destroying the metal centers housed therein. Once accomplished, large quantities of the metallosubunits could be isolated and spectroscopically studied. We report here that

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* To whom correspondence should be addressed.

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[1] Abbreviations: CODH, carbon monoxide dehydrogenase from *Clostridium thermoaceticum*; CoA, coenzyme A; SDS, sodium dodecyl sulfate; BIS, methylenebis(acrylamide); DTT, dithiothreitol; TEMED, *N,N,N′N′*-tetramethylethylenediamine; AP, 1% freshly-prepared ammonium persulfate.

[2] We previously called the A-cluster "the NiFe complex" but have changed nomenclature to unify literature in this area. The "A" highlights the importance of this cluster for acetyl-CoA synthesis.

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CODH treated with low concentrations of the detergent sodium dodecyl sulfate (SDS) and then subjected to electrophoresis decomposes into brown-colored α metallosubunits and a brown-colored, catalytically-active species consisting predominantly of β metallosubunits.

EXPERIMENTAL PROCEDURES

C. thermoaceticum was grown, and CODH was purified and characterized as described (Lundie & Drake, 1984; Ragsdale & Wood, 1985; Raybuck et al., 1988; Ramer et al., 1989; Shin & Lindahl, 1993). The enzyme had CO oxidation and CO/acetyl-CoA exchange activities of 390 and 0.13 units/mg, respectively. Protein concentrations were determined by the biuret method (Pelley et al., 1978) using bovine serum albumin as a standard.

Electrophoresis. Discontinuous polyacrylamide gel electrophoresis was performed essentially as described (Andrews, 1986), except for differences that arose because it was performed anaerobically in a glovebox (Vacuum/Atmospheres model HE453). All solutions were degassed using Schlenk techniques. The separating solution (6.0 mL) was prepared in the box by combining 3.0 mL of H_2O, 0.90 mL of 2 M Tris·Cl, pH 8.8, 2.1 mL of monomer stock solution (18% acrylamide, 0.48% BIS), 18 μL of 1 M DTT, and 4 μL TEMED. The stacking solution (1.6 mL) was prepared simultaneously by combining 1 mL of H_2O, 0.25 mL of 1 M Tris·Cl, pH 6.8, 0.35 mL of monomer stock solution, 6 μL of 1 M DTT, and 3 μL of TEMED.

The lower opening between the electrophoresis plates (Hoefer Scientific model SE250) was sealed with tape and then layered with 0.1 mL of 1% freshly-prepared ammonium persulfate (AP) and 0.5 mL of the separating solution. The solution gelled within 3 min. One-half microliter of AP was added to 1 mL of the separating solution. If this solution gelled in 3−5 min, 2 μL AP was added to the remaining 4.5 mL of separating solution. If the test solution did not gel, or if it gelled too rapidly, the amount of AP added to the remaining solution was adjusted accordingly. The solution was immediately poured between the electrophoresis plates. After 40−50 min, the stacking solution was mixed with 5 μL of AP, and 1.2 mL was poured over the separating gel. The Teflon comb was inserted as soon as the unused stacking solution began to gel.

CODH samples (10 μL of a 7.7 mg/mL stock frozen directly after the final purification column) were prepared for electrophoresis by incubating with 1 μL of 1 M DTT for 10 min at 26 °C. Between 0 and 14 μL of any of three SDS stock solutions (34.6, 346, or 867 mM SDS prepared in 50 mM Tris, pH 8.0) and 14 and 0 μL of 50 mM Tris, pH 8.0, were then added, and the resulting 25 μL solutions were incubated for 1 h at temperatures ranging from 5 to 26 °C. Immediately before loading, samples were mixed with 15 μL of 40% glycerol and a trace of bromophenol blue (BioRad).

At least 30 min after the stacking gel was poured, the comb and the tape were removed and samples were loaded. Electrode buffer (25 mM Tris base, 0.192 M glycine) covered electrodes and plates. The upper buffer also contained 3 mM DTT. A potential (2 W constant power) was applied (BioRad Power-Pac 3000) until the dye front reached the bottom of the gel (typically 50 min).

For activity staining, the top plate of the gel sandwich was removed and CO oxidation assay solution (50 mM Tris, pH 8.0, 10 mM methyl viologen) was poured on the gel surface. After 10 min, excess solution was decanted and the gel was transferred to an air-tight plexiglass container. The gel−container assembly was removed from the glovebox, exposed to CO, and photographed six times at 10 s intervals. The gel was subsequently stained for protein, using Coomassie blue G250 (BioRad).

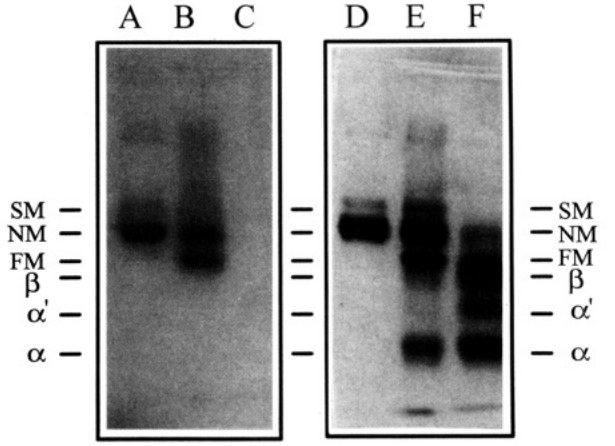

FIGURE 1: One-dimensional native polyacrylamide gel electrophoresis of CODH. The gel was first stained for activity and photographed 75 s after CO exposure (lanes A−C) and then stained for protein (D−F). A and D, 5.7 μg of native CODH; B and E, 9.6 μg of CODH, incubated in 1600 equiv/$\alpha_3\beta_3$ of SDS at *ca.* 7 °C; C and F, 9.6 μg of CODH, incubated in 28 000 equiv/$\alpha_3\beta_3$ of SDS at *ca.* 7 °C.

Two-dimensional gels were performed aerobically. Unstained vertical strips of protein-impregnated gels were incubated in 62.5 mM Tris·Cl, pH 6.8, 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol for 30 min at 70 °C. Each strip was then placed horizontally on top of a standard SDS−polyacrylamide staking gel (Laemmli, 1970), prepared without a comb, and sealed with agarose. Samples were electrophoresed at room temperature using a 30 mA constant current and stained as above. Densities of electrophoretic regions were integrated using a Molecular Dynamics computing densitometer, model 300A.

RESULTS

Native CODH was electrophoresed through a polyacrylamide gel under anaerobic conditions, as described in the Experimental Procedures. Afterward, the gel was soaked in a solution containing oxidized methyl viologen and exposed to a CO atmosphere. One dark band of reduced methyl viologen developed (Figure 1A), indicating a catalytically-active species that is almost certainly native enzyme ($\alpha_3\beta_3$). We refer to the electrophoresed species in the gel as NM-CODH (native-migrating) rather than native CODH, a term we reserve for CODH prior to electrophoresis. The small amount of diffuse activity above the NM-CODH band was designated a second catalytically-active band called SM-CODH (slower-migrating).

The gel was then stained for protein and one dark protein band obtained (Figure 1D) at the location corresponding to the NM-CODH activity band. A minor protein band, corresponding to SM-CODH, was present as well. The small proportion of protein in the SM-CODH form suggests that little of this form was present in native enzyme solutions.

An equivalent gel strip was soaked in 2% SDS-containing sample buffer, heated at 70 °C, and electrophoresed in a

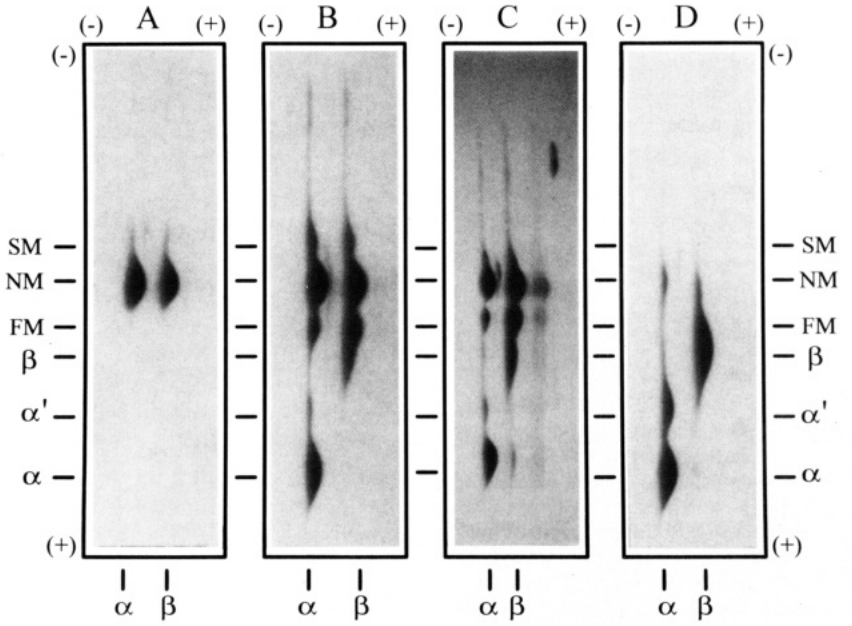

FIGURE 2: Two-dimensional electrophoretic gels of CODH. A, equivalent to samples A and D of Figure 1; B, equivalent to samples B and E of Figure 1; C, 10.5 μg of CODH (from a batch with 260 units/mg CO oxidation activity) incubated in 740 equiv/$\alpha_3\beta_3$ of SDS and 100 mM 2-mercaptoethanol for 1 h at *ca.* 26 °C; D, equivalent to samples C and F of Figure 1.

second dimension through a gel containing 0.08% SDS. This treatment served to separate and denature the subunits. Two protein regions were obtained upon staining (Figure 2A), with integrated densities of 53% and 47%. These regions corresponded to the denatured α and β subunits of NM-CODH, respectively. The slightly unequal integrated densities arise from differences in the molecular mass of the subunits and because Coomassie blue appears to stain α approximately 8% darker than β (average of six trials).

Another sample of CODH was incubated with 1600 equiv/$\alpha_3\beta_3$ of SDS at 7 ± 2 °C, and then subjected to electrophoresis as described in the Experimental Procedures. Activity staining of the resulting gel (Figure 1B) yielded two major activity bands of approximately equal intensity. One band corresponded to NM-CODH, while the other was a new band that migrated faster than NM-CODH, called FM-CODH (faster-migrating). A small amount of SM-CODH was also present. Protein staining (Figure 1E) followed by densitometry analysis revealed that SM-CODH, NM-CODH, and FM-CODH represented 11%, 55%, and 20% of the total protein in the gel, respectively. The gel contained a fourth major band, 14% of total protein, which corresponded to the α subunit (see below). α was devoid of catalytic activity and migrated faster than either NM-CODH or FM-CODH. The gel also contained two diffuse minor bands, labeled β and α′, that could not be accurately quantified. β migrated slightly faster than FM-CODH, and α′ migrated slightly slower than α.

The composition the these bands was evaluated by two-dimensional electrophoresis (Figure 2B). The resolution of two-dimensional gels is poorer than that of one-dimensional gels, but the β/α ratios for SM-CODH, NM-CODH, and FM-CODH were estimated from the integrated densities of spots to be approximately 1.0, 1.2, and 2.7, respectively (after accounting for the slightly different staining properties of α and β). Substituting 2-mercaptoethanol for dithiothreitol during the incubation step improved the resolution of the bands (but yielded somewhat less FM-CODH). A two-dimensional gel prepared with 2-mercaptoethanol (Figure 2C) exhibited FM-CODH regions with a β/α ratio also of 2.7. Because small amounts of β may have overlapped FM-CODH somewhat, the observed 2.7 β/α ratio for FM-CODH may be greater than the actual ratio of the molecule by as much as ~30%. We conclude that the actual β/α ratio for FM-CODH is between 2 and 3.

The bands called α and α′ were different forms of the α subunit (Figure 2B,C). When gels were overloaded, the α form was found to be brown (not shown), indicating that it contains Fe-S clusters. We show below that α′ represents denatured α. Under the mild conditions of this experiment, the α metallosubunit predominates over the α′ denatured form.

We monitored the optical density of NM-CODH and FM-CODH activity bands with time after exposure to CO and divided these values by the integrated densities of the corresponding protein bands. We call this ratio the "specific activity" of a band. As shown in Figure 3, FM-CODH had essentially twice the specific activity of NM-CODH. This result can be visually verified by comparing the activity bands in Figure 1B (NM-CODH and FM-CODH have about equal intensities) to the corresponding protein bands in Figure 1E (NM-CODH is about 2 times as intense as FM-CODH).

Another sample of CODH was incubated in a higher concentration of SDS (28 000 equiv/$\alpha_3\beta_3$) and electrophoresed as before. In this case, no bands in the resulting gel had catalytic activity (Figure 1C). Protein staining (Figure 1F) revealed four protein bands, including the β subunit (46%), α′ (15%), α (33%), and a band that migrated similarly to NM-CODH (6%). Since this last species had no catalytic activity (Figure 1C), it cannot be NM-CODH, and we label it NM′. These assignments were verified from the two-dimensional gel protein regions (Figure 2D). The proportion of α′ relative to α increased under these conditions, indicating that SDS at higher concentrations denatured a larger proportion of α subunits.

Other samples of CODH were incubated in various intermediate concentrations of SDS, ranging from 800 to

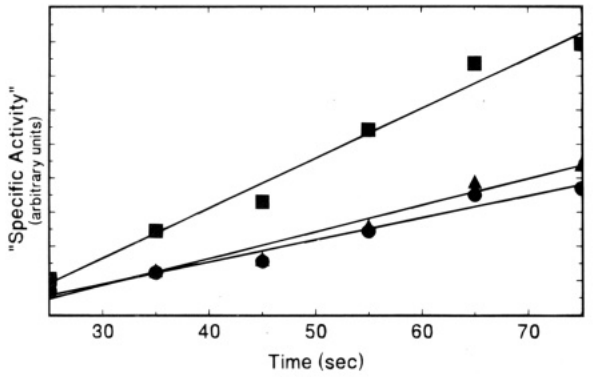

FIGURE 3: CO oxidation activity of NM-CODH and FM-CODH. The integrated densities of CO oxidation activity bands were monitored with time after samples were exposed to CO. "Specific activities" are defined here as the integrated densities of the activity divided by that of the corresponding protein band. Activity densities for the 75 s point are shown in Figure 1 A,B. Squares, FM-CODH (Figure 1B); triangles, NM-CODH from SDS-treated enzyme (Figure 1B); circles, NM-CODH from native enzyme (Figure 1A). Solid lines through the data are least-squares best-fit lines. The ratio of slopes for squares/triangles equals 1.9; for squares/circles, the ratio is 2.3.

78 000 equiv/mol, and electrophoresed to observe the proportions of the various disassociation products (Figure 4). The only species observed were SM-CODH, NM-CODH, FM-CODH, β, α, α', and NM′. The catalytically-active species, SM-CODH, NM-CODH, and FM-CODH, were present in greatest proportions at low SDS concentrations. As the SDS concentration increased, their intensities declined and the intensities of individual α and β subunits increased. Importantly, no other decomposition products ($\alpha_2\beta_3$, $\alpha_3\beta_2$, $\alpha_3\beta_1$, etc.) were observed. The α metallosubunit was quite stable over a wide range of SDS concentrations. However, incubation in high concentrations of SDS (78 000 equiv/$\alpha_3\beta_3$) at high temperatures (70 °C) transformed α quantitatively to the denatured α' form (Figure 4I). Surprisingly, even after these harsh conditions, samples still retained some brown color, suggesting that all or some fraction of α' and/or β contained at least one Fe-S cluster.

DISCUSSION

The subunits of CODH are held together by noncovalent forces such as hydrophobic and/or electrostatic interactions. SDS disrupted these interactions and separated the subunits. When CODH was incubated at high temperatures in high concentrations of SDS, the subunits dissociated from each other and denatured. The α and β bands in the two-dimensional gel of Figure 2A were obtained under such conditions.

The aim of this study was to use less severe SDS conditions to separate CODH into simpler forms that retained their metal centers. Conditions were found that yielded three catalytically-active species, called SM-CODH, NM-CODH, and FM-CODH. Overloaded gels reveal that these species were brown in color, indicating the presence of iron−sulfur clusters. These forms must contain all of the metal centers required for CO oxidation catalysis. The number of each type of subunit in these species was estimated using the β/α ratios for SM-CODH, NM-CODH, and FM-CODH (1.0, 1.2, and 2.7, respectively) and the assumption that the ratios were composed of small integers. Since NM-CODH migrated at the same rate as native CODH, had essentially the same specific activity as native CODH, and had approximately equal numbers of α and β subunits, NM-CODH appears to be native enzyme ($\alpha_3\beta_3$). Since FM-CODH had a β/α ratio between 2 and 3, and it migrated faster than NM-CODH, it probably has either an $\alpha_1\beta_3$ or $\alpha_1\beta_2$ quaternary structure. Establishing its quaternary structure will require an analytical centrifugation study of isolated FM-CODH. The quaternary structure of SM-CODH is similarly uncertain. SM-CODH exhibited a β/α ratio of 1, suggesting equal numbers of subunits. We considered $\alpha_2\beta_2$ and $\alpha_1\beta_1$ structures, but SM-CODH migrated *slower* than NM-CODH, suggesting either more subunits (e.g., $\alpha_4\beta_4$) or the same number of subunits but a larger size or smaller charge.

Since no other decomposition products (such as $\alpha_2\beta_3$, $\alpha_3\beta_2$, or $\alpha_3\beta_1$) were observed at any concentration of SDS, such species are probably unstable. The molar yield of FM-CODH was between 24% and 31% for the experiments reported here. Higher yields have very recently been obtained using samples of CODH devoid of NaCl. The yield appears to result from two competing processes: NM-CODH decomposing into FM-CODH and FM-CODH decomposing into individual subunits. These processes depend on SDS concentration and temperature; *ca.* 1600 equiv/$\alpha_3\beta_3$ of SDS and 7 °C appear optimal. Lower concentrations and temperatures appear to inhibit the decomposition of NM-CODH into FM-CODH, while higher concentrations and temperatures promote the decomposition of FM-CODH into subunits.

Why should some subunits (2 α's and 0−1 β) so readily dissociate from the native $\alpha_3\beta_3$ structure, while the remaining

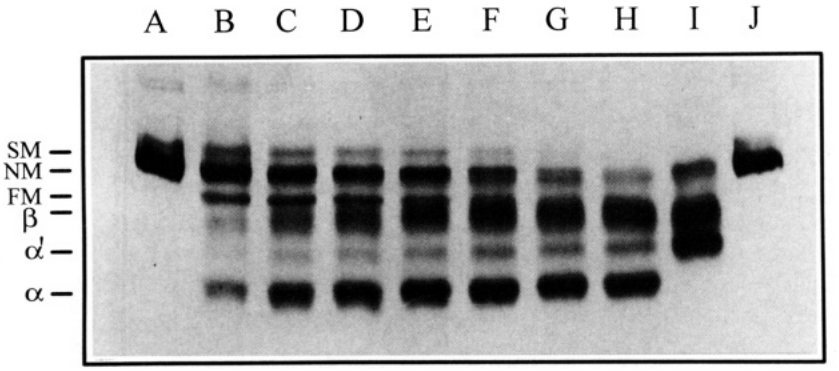

FIGURE 4: Electrophoretic gel of CODH incubated in various amounts of SDS. A and J, 9.6 μg of native CODH; B−H, same as lanes A and J, except incubated for 1 h at 13 °C in the following numbers of equiv/$\alpha_3\beta_3$ of SDS: B, 830; C, 1700; D, 3100; E, 6300; F, 15 000; G, 31 000; H, 78 000 (some of the SDS in H precipitated from solution); I, same as lane H, except heated at 70 °C for 0.5 h. The gel was 6.7% polyacrylamide.

subunits do not? One possibility is that the dissociable subunits have weaker intersubunit attractive forces than the nondissociable subunits. The implication of this is that the α (and maybe β) subunits are heterogeneous, consistent with the proposal of Shin et al. (1993) that one αβ unit differs from the other two (they presented evidence that each $\alpha_3\beta_3$ molecule contains only one A-cluster). Another possibility is that all subunits bind with equal strength and that FM-CODH results from the random dissociation of subunits. Since this possibility leaves unanswered why other decomposition products were not also observed, we favor the former possibility.

If the molecular mass of FM-CODH were known, its CO oxidation turnover frequency could be compared to that of NM-CODH by multiplying their respective specific activities by their molecular masses. Assuming $\alpha_1\beta_2$ and $\alpha_1\beta_3$ structures for FM-CODH yields turnover frequencies equal to or 30% faster than NM-CODH, respectively. The similarity of turnover frequencies suggests that (i) removing the dissociable subunits does not adversely affect the CO oxidation activity of the remaining species, (ii) the dissociable subunits are not required for CO oxidation activity, and (iii) the clusters required for CO oxidation are located in the nondissociable subunits. These clusters appear to include the active-site C-cluster (Anderson et al., 1993) and one or more Fe_4S_4 clusters, which function to transfer electrons between the C-cluster and the external redox agents (Anderson & Lindahl, 1994; Kumar et al., 1994). Other unidentified clusters may be involved, as well.

CODH from *Rhodospirillum rubrum* is a monomer that catalyzes the oxidation of CO but not the synthesis of acetyl-CoA (Bonam & Ludden, 1987; Ensign et al., 1989). It contains a C-cluster-like active site as well as Fe-S clusters (Stephens et al., 1989). Since its amino acid sequence is 46% identical (67% similar) to the β subunit of the CODH from *C. thermoaceticum* (Kerby et al., 1992), we suspect that the CO oxidation apparatus, including the C-cluster and one or more Fe_4S_4 clusters, is located in β.

The α subunits appear to be involved in the other catalytic function of the enzyme, acetyl-CoA synthesis. Two of the other proteins required for this activity bind to α. The corrinoid/iron−sulfur enzyme, which functions to transfer the methyl group to CODH, appears to be linked *in-vivo* to α cysteine-506 of CODH *via* a disulfide bond (Shanmugasundaram et al., 1993). The ferredoxin that stimulates acetyl-CoA synthesis appears to dock at residues 229−239 of the α subunit of CODH (Shanmugasundaram & Wood, 1992). Moreover, CoA binds to the α subunit. The adenine portion is associated with α tryptophan-418 (Shanmugasundaram et al., 1988; Morton et al., 1991), while the pyrophosphate portion is thought to interact with a nearby arginine residue on α (Shanmugasundaram et al., 1989). Interestingly, there is also evidence for heterogeneous CoA binding to CODH. A Scatchard plot fits best to two CoA binding sites (K_d = 52 μM for one site and 2600 μM for the other) (Lu & Ragsdale, 1991). Since K_m = ~50 μM for this substrate (Ramer et al., 1989; Lu & Ragsdale, 1991), only the tightly bound CoA appears to function catalytically.

The β subunit may also be involved in the synthesis of acetyl-CoA. The methyl group used to synthesize acetyl-CoA binds a cysteine residue on β upon acidification of methylated enzyme (Pezacka & Wood, 1988). The methyl may have migrated to this cysteine from an active-site metal (Lu et al., 1990). Given the evidence that the A-cluster is the active site for this reaction, the reactive cysteine and this cluster are probably in close proximity. The sulfhydryl portion of CoA is probably located near the A-cluster as well, since this moiety is directly involved in catalysis (Raybuck et al., 1988). Congruent with this idea is evidence that CoA alters the EPR signal from the A-cluster (Ragsdale et al., 1985). The length of the CoA molecule would allow those sites on α that bind the nucleotide and phosphate portions of CoA to be some distance from the A-cluster. Thus, the A-cluster might be in either α or β.

It will be important to measure the acetyl-CoA synthase activity of α and FM-CODH once large quantities of these species are available. Even more important may be to examine their spectroscopic properties. Such studies should clarify the distribution of metal centers in the subunits and the additional structural features of these centers. Taken together, the kinetic and spectroscopic properties of these species should dramatically improve our understanding of how the metal centers in this extremely-complicated enzyme function during catalysis. Such studies are underway.

NOTE ADDED IN PROOF

Very recent analytical centrifugation experiments indicate that FM-CODH has an $\alpha_1\beta_2$ quaternary structure and, unexpectedly, that native CODH has an $\alpha_2\beta_2$ structure. These studies and their implications will be reported shortly.

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