Carbon Monoxide Dehydrogenase from *Clostridium thermoaceticum*: Quaternary Structure, Stoichiometry of Its SDS-Induced Dissociation, and Characterization of the Faster-Migrating Form†

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Received May 26, 1995; Revised Manuscript Received October 19, 1995⊗

ABSTRACT: The molecular mass (M_r) of the nickel- and iron-sulfur-containing enzyme CO dehydrogenase from *Clostridium thermoaceticum* was determined by sedimentation equilibrium ultracentrifugation to be 300 000 ± 30 000 Da. Since the enzyme is known to contain equal numbers of two types of subunits (M_r = 82 000 Da for α and 73 000 Da for β), this indicates an $\alpha_2\beta_2$ quaternary structure. The enzyme was previously thought to have an $\alpha_3\beta_3$ structure because it migrates through calibrated size-exclusion chromatographic columns with an apparent M_r of about 420 000 Da. The disproportionately fast migration rate suggests that the enzyme is nonspherical. SDS induces the dissociation of an α subunit, yielding a stable species called FM-CODH. FM-CODH had a molecular mass of 210 000 ± 30 000 Da, indicating an $\alpha_1\beta_2$ structure. It contained 2.1 ± 0.3 Ni and 16 ± 3 Fe per $\alpha_1\beta_2$, exhibited S → Fe charge-transfer transitions typical of Fe-S proteins, and afforded the g_{av} = 1.82, 1.86, and 1.94 EPR signals. Quantitation of the 1.82 and {1.94 + 1.86} signals afforded 0.35 and 1.9 spin/$\alpha_1\beta_2$, respectively. FM-CODH samples exhibited CO oxidation activity, but little CO/acetyl-CoA exchange activity. Some FM-CODH samples exhibited CO oxidation activities as high as native enzyme. These results, along with the quantified spin intensities of the EPR signals, indicate that FM-CODH contains the B- and C-clusters and suggest that these clusters are located in the β subunit. The α subunit that dissociated during formation of FM-CODH is not required for CO oxidation activity. FM-CODH is either devoid of A-clusters, or if such clusters are present, they have lost their ability to exhibit substantial NiFeC signals and CO/acetyl-CoA exchange activity. Incubating FM-CODH and α yielded a species that migrated through polyacrylamide gels at the same rate as native enzyme, and had a molecular mass indicating an $\alpha_2\beta_2$ structure. Thus, the SDS-induced dissociation of the enzyme appears to be reversible.

Carbon monoxide dehydrogenases are one of only four types of naturally-occurring Ni-containing enzymes. The enzymes found in acetogenic bacteria serve a central role in a metabolic pathway that allows these bacteria to grow autotrophically on either CO or CO_2/H_2 (Ragsdale, 1991; Wood & Ljungdahl, 1991). Besides catalyzing the reversible oxidation of CO to CO_2, the enzyme from *Clostridium thermoaceticum* ($CODH_{Ct}$[1]) catalyzes the synthesis of acetyl-CoA from CO, coenzyme A, and a methyl group originating from methyltetrahydrofolate.

The molecular mass and quaternary structure of $CODH_{Ct}$ have been the subject of some controversy. Drake et al. (1980) and Ragsdale et al. (1983b) obtained molecular masses of 410 000 and 436 000 Da, respectively, using calibrated gel-filtration chromatography. Ragsdale et al. (1983b) found that the enzyme had two types of subunits, α and β, present in approximately equal amounts on SDS−PAGE gels. From their approximate molecular masses, [later determined to be 81 730 and 72 928 Da, respectively (Morton et al., 1991)], Ragsdale et al. (1983b) concluded that $CODH_{Ct}$ had an $\alpha_3\beta_3$ quaternary structure. Diekert and Ritter (1983) independently purified the enzyme and found that it contained two subunits as well, but obtained a molecular mass of 250 000 Da using gradient gel electrophoresis. They did not propose a quaternary structure. Subsequently, Diekert (1988) obtained a molecular mass of 290 000 Da for the enzyme, using sucrose-gradient centrifugation, and concluded that the enzyme had an $\alpha_2\beta_2$ quaternary structure. A year later, Ramer et al. (1989) used calibrated gel-filtration chromatography to obtain a molecular mass of 410 000 Da for enzyme with high CO/acetyl-CoA exchange activity. They concluded that enzyme with acetyl-CoA synthase activity had an $\alpha_2\beta_2\gamma_2$ structure, because SDS−PAGE gels of their preparations exhibited three bands of approximately equal intensity (with molecular masses of 78 000, 71 000, and 50 000 Da). However, Lu and Ragsdale (1991) subsequently demonstrated that the proposed 50 000 Da "γ subunit" was unnecessary for any known catalytic activity of the enzyme. Moreover, Roberts et al. (1989) identified a cluster of genes in the *Clostridium thermoaceticum* genome coding for the

† This research was supported by the National Institutes of Health (GM46441), the Office of Naval Research (N00014-93-1-0991), and the Robert A. Welch Foundation (A865 and A1170).

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⊗ Abstract published in *Advance ACS Abstracts,* January 1, 1996.

[1] Abbreviations: $CODH_{Ct}$, carbon monoxide dehydrogenase from *Clostridium thermoaceticum*; SDS, sodium dodecyl sulfate; FM-CODH, faster-migrating form of $CODH_{Ct}$; BIS, methylenebis(acrylamide); DTT, dithiothreitol; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; PAGE, polyacrylamide gel electrophoresis; EPR, electron paramagnetic resonance; SD, standard deviation.

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α and β subunits of $CODH_{Ct}$ and for other proteins used in the synthesis of acetyl-CoA, but not for the postulated γ subunit.

In spite of the unsettled nature of this issue, we and many others in the field have assumed that the quaternary structure of $CODH_{Ct}$ was $\alpha_3\beta_3$, not $\alpha_2\beta_2$. The sample used by Diekert and Ritter (1983) was neither as active nor as pure as that used by Ragsdale et al. (1983b), and few experimental details were included in the chapter of the book describing the sucrose-gradient centrifugation study (Diekert, 1988). Moreover, the $\alpha_3\beta_3$ proposal reinforced a hypothesis used to explain some of the unusual properties of the enzyme's Ni and Fe-S centers. One of these centers, called the *A-cluster*, consists of a Ni complex bridged to an Fe-S cluster, and is almost certainly the active site for acetyl-CoA synthesis (Ragsdale et al., 1982, 1983a, 1985; Bastian et al., 1988; Lindahl et al., 1990a,b; Fan et al., 1991; Gorst & Ragsdale, 1991; Shin & Lindahl, 1992a,b). When reduced by 1 e^- and bound with CO, the A-cluster is $S = {}^1/_2$ and yields the so-called NiFeC EPR signal. For an unknown reason, the maximum intensity of this signal is only ~0.3 spin/αβ, or about one-third of that expected. The Ni in the A-cluster can be removed with 1,10-phenanthroline, and reinserted by incubating the phenanthroline-treated enzyme in $NiCl_2$ (Shin & Lindahl, 1992a,b; Shin et al., 1993). Shin et al. (1993) found that only about 0.3 Ni/αβ was removed/reinserted in this process. This result, in conjunction with the low spin intensity of the NiFeC signal and the $\alpha_3\beta_3$ quaternary structure of $CODH_{Ct}$, suggested that only one αβ dimer per hexameric molecule of $CODH_{Ct}$ contained an A-cluster.

Another Ni-Fe-S cluster in the enzyme, called the *C-cluster*, is probably the CO oxidation active site (Anderson et al., 1993; Kumar et al., 1993; Anderson & Lindahl, 1994; Qiu et al., 1995). It is diamagnetic when oxidized, and $S = {}^1/_2$ when reduced by one electron ($E^{o\prime} = -220$ mV). The reduced state exhibits an EPR signal with $g_{av} = 1.82$. Upon further reduction of the enzyme, the $g_{av} = 1.82$ signal disappears, and is replaced by another signal with $g_{av} = 1.86$ (Lindahl et al., 1990a). The catalytic function of this conversion and the relationship between the $C_{1.82}$ and $C_{1.86}$ states are unclear; $C_{1.86}$ may be two electrons more reduced than $C_{1.82}$, or the two states may be isoelectronic (Anderson & Lindahl, 1994). The C-cluster was thought to be present in only one αβ dimer per hexamer, again because of the low intensity of the $g_{av} = 1.82$ and $g_{av} = 1.86$ signals (Shin et al., 1993).

The only other type of metal cluster in the enzyme, an $[Fe_4S_4]^{2+/1+}$ cluster called the *B-cluster*, yields a $g_{av} = 1.94$ signal that quantifies to 0.64 ± 0.14 spin/αβ (Lindahl et al., 1990a). This cluster appears to transfer electrons between the C-cluster and external redox agents (Anderson & Lindahl, 1994; Kumar et al., 1993).

We recently discovered that $CODH_{Ct}$ treated with small amounts of the detergent SDS dissociates into α metallosubunits and a species called *FM-CODH* that migrates faster than native enzyme on nondenaturing polyacrylamide gels (Xia & Lindahl, 1995). Isolated α contains one Ni and four Fe (Xia et al., 1995). The Ni appears to be coordinated by two S and two N/O donors, in a distorted square-planar geometry, and the irons are organized into an $[Fe_4S_4]^{2+/1+}$ cluster that is $S = {}^3/_2$ when reduced. The faster-migrating form of $CODH_{Ct}$, when embedded in polyacrylamide gels, is as active as native $CODH_{Ct}$ in catalyzing CO oxidation. Although the quaternary structure of FM-CODH was not established, the observed β/α ratio of 2−3 suggested either an $\alpha_1\beta_3$ or an $\alpha_1\beta_2$ structure.

We have now isolated FM-CODH in large quantities, and determined its metal content, quaternary structure, catalytic abilities, UV−vis, and EPR properties. These properties were used to determine the metal clusters in FM-CODH. The quaternary structure of native $CODH_{Ct}$ was determined using analytical ultracentrifugation, and the stoichiometry and reversibility of the reaction induced by SDS were established.

EXPERIMENTAL PROCEDURES

Preparation and Characterization of CODH. Three batches of $CODH_{Ct}$ were purified from *Clostridium thermoaceticum*, using a published procedure (Shin & Lindahl, 1993). All batches were ~90% pure according to SDS−PAGE. Batches 1, 2, and 3 had CO oxidation activities of 200, 240, and 260 units/mg and CO/acetyl-CoA exchange activities of 0.05, 0.03, and 0.13 units/mg, respectively. Protein concentrations were determined by the biuret method (Pelley et al., 1978), and CO oxidation activities were determined as described (Ragsdale et al., 1983b; Shin & Lindahl, 1993). Metal concentrations were determined by the standard curve method using an atomic absorption spectrophotometer (Perkin-Elmer Model 2380). Analytical nondenaturing and SDS−polyacrylamide gels were prepared as described (Xia & Lindahl, 1995). Electrophoretic regions were integrated using a computing densitometer (Molecular Dynamics, Model 300A). The EPR spectrometer and spin quantitation method used have been described (Shin et al., 1992; Roberts & Lindahl, 1995).

Isolation of FM-CODH. The following procedures were performed in an argon atmosphere glovebox (Vacuum/Atmospheres HE453) with ~1 ppm of O_2, monitored continuously (Teledyne Model 310). All solutions were rendered anaerobic by lengthy degassing on a Schlenk line. The 8% polyacrylamide separating gel was prepared as follows: to 4.11 g of a 37.1 g of acrylamide/1 g of BIS mixture (BioRad) were added 5.2 mL of 2 M Tris·HCl, pH 8.8, 42 mL of H_2O, 0.15 mL of 1 M DTT, and 20 μL of TEMED. The solution was degassed for 5 min, mixed with 35 μL of 0.5% (w/v) fresh ammonium persulfate, and poured into a stand-mounted 37 mm i.d. gel tube of a preparative electrophoresis cell (BioRad, Model 491). After the gel solidified (10 min), the top was layered with 0.2 M Tris, pH 8.8. The gel polymerized completely overnight.

The 4% polyacrylamide stacking gel was prepared as follows: To 0.8 g of the same acrylamide/BIS mixture were added 3.1 mL of 1 M Tris·HCl, pH 6.8, and 16 mL of H_2O. This stock solution was frozen until needed. To 6 mL of stock were added 5 μL of TEMED and 5 μL of 0.5% fresh ammonium persulfate. The buffer was removed from the top surface of the polymerized separating gel, and was replaced by the stacking-gel solution.

Samples were prepared during the 30 min required for the stacking gel to polymerize. Between 2.6 and 3.9 mL of 7.7 mg/mL native $CODH_{Ct}$ (directly from the Sephacryl S-300 purification column) was concentrated with a Centricon (Amicon, Inc.) to about 0.5 mL. To this solution were added 2.5 mL of 50 mM Tris·HCl, pH 8, and 0.3 mL of 1 M DTT. The resulting solution was cooled to 1−4 °C, mixed gently

Table 1: Molecular Masses and CO Oxidation Activities of Native $CODH_{Ct}$ and SDS-Induced Dissociation Products

batch	species	concentrations[a] (mg/mL)	buffer	S.A. before (after)[b] (units/mg)	molecular mass[c] (Da)
1	native	5.4; 2.7; 1.4	50 mM Tris, pH 8, 0.2 M NaCl, 2 mM $S_2O_4^{2-}$, 1 mM DTT	220 (200)	300 000 ± 20 000
1	native	2.7; 1.4; 0.7	50 mM Tris, pH 8, 0.2 M NaCl, 2 mM $S_2O_4^{2-}$, 1 mM DTT	240 (220)	310 000 ± 20 000
3	FM	12	192 mM glycine, 25 mM Tris, pH 8, 2 mM $S_2O_4^{2-}$, 3 mM DTT	360, 290	not determined
2	FM	4; 2; 1.3	25−100 mM glycine, 37−47 mM Tris, pH 8, 1 mM $S_2O_4^{2-}$, 1 mM DTT	220 (160)	210 000 ± 40 000
1	FM	*X*; 0.65*X*; 0.35*X*	210 mM NaCl, 50 mM Tris, pH 8, 1.8 mM $S_2O_4^{2-}$, 1.8 mM DTT	200 (ND)	230 000 ± 20 000
1	FM	7.7; 5.8; 3.9	192 mM glycine, 25 mM Tris, pH 8, 2 mM $S_2O_4^{2-}$, 3 mM DTT	80 (6)	210 000 ± 10 000
1	FM	6.9; 5.8; 3.9	192 mM glycine, 25 mM Tris, pH 8, 10 mM $S_2O_4^{2-}$, 5 mM DTT	110 (110)	180 000 ± 10 000
1	FM	6; 3; 1.2	192 mM glycine, 25 mM Tris, pH 8, 10 mM $S_2O_4^{2-}$, 5 mM DTT	150 (140)	220 000 ± 10 000
1	α	*X*; 0.57*X*; 0.42*X*	192 mM glycine, 25 mM Tris, pH 8, 2 mM $S_2O_4^{2-}$, 1 mM DTT		80 000 ± 10 000
1	α	3; 1.5; 0.75	192 mM glycine, 25 mM Tris, pH 8, 20 mg/mL Triton X-100		100 000 ± 4000
1	recon	1 α; 3.4 FM	192 mM glycine, 25 mM Tris, pH 8, 20 mg/mL Triton X-100, 5 mM DTT	130 (130)	280 000 ± 20 000

[a] Concentrations of ultracentrifugation samples; those listed as X were not determined. [b] Specific activities were determined before and after ultracentrifugation, to assess whether oxidative damage had occurred during sample handling. The few samples that suffered significant activity loss were not included in the table. [c] The molecular masses reported here are the average of the three trials associated with each experiment, and the relative uncertainties are the corresponding standard deviations. The relative uncertainties in the goodness-of-fit parameter for individual data-sets were substantially less than the listed deviations. On the other hand, the estimated overall relative uncertainties associated with the molecular masses of FM, α, and the reconstituted enzyme (±15%) are larger than the listed deviations, because they include uncertainties in the number of detergent molecules bound, partial specific volumes, buffer densities, possible nonideal sedimentation effects, and uncertainties arising from differences among individual samples. Given that native $CODH_{Ct}$ was not exposed to detergent or subjected to electrophoresis, the overall relative uncertainty associated with its molecular mass determination is estimated at ±10%.

with 0.9−1.3 mL of 10% (w/v) SDS, and incubated 45−60 min.

The lower reservoir of the Prep cell was filled with 0.025 M Trizma base, 0.192 M glycine, and 6 mM dithionite. The same buffer was used in the upper reservoir, except that it lacked dithionite, contained 3 mM DTT, and was precooled to about 15 °C. The buffer in the lower reservoir was chilled to about 15 °C by passing it through a heat-exchanger. The ceramic cooling rod forming the core of the gel was cooled similarly.

After incubation, the sample was mixed with 1.5 mL of 80% (w/w) glycerol, and loaded on the polymerized stacking gel surface. Approximately 3 h after applying 12 W constant power, the cell was disassembled, and the cylindrically-shaped, protein-impregnated gel was transferred from the cooling rod to a plexiglass rod. Protein bands were sliced with a blade from the gel using an apparatus in which the rod/gel assembly was rotated around the rod axis.

The membrane caps of a BioRad Model 422 Electro-eluter were attached to their silicone adapters, filled with 0.025 M Trizma base, 0.192 M glycine, 3 mM DTT, and 6 mM dithionite, and attached to the fitted ends of glass tubes already assembled in the eluter. Excised protein bands were cut into small pieces and loaded into the tubes. The tubes were filled with 0.2 M Tris·HCl, pH 6.8, and 3 mM DTT. The lower and upper reservoirs of the eluter contained the same buffers used in the analogous reservoirs of the Prep cell. Power (10 W; 2 W per tube × 5 tubes) was applied for 1−2 h. FM-CODH was collected above the cap membranes.

Recombination of FM-CODH and α. At room temperature, 1 μL of 1 M DTT, 10 μL of 50 mM Tris, pH 8, 2 μL of 200 mg/mL Triton X-100 (from Sigma, prepared in water), 2 μL of α (5.2 mg/mL), and 5 μL of FM-CODH (6 mg/mL) were mixed, in that order, in an Eppendorf tube, and incubated for 1 h. α was isolated as described (Xia et al., 1995), and used immediately. α prepared with the eluter or stored for days prior to use afforded multiple bands on nondenaturing gels (possibly reflecting a tendency of α to aggregate) and lower yields of the reconstituted enzyme. For electrophoresis studies, samples were mixed with 15 μL of 80% (w/w) glycerol just prior to loading onto gels.

Analytical Ultracentrifugation. Experiments were performed with native $CODH_{Ct}$, FM-CODH, α, and the reconstituted enzyme. The individual experiments performed, and the buffers used, are given in Table 1. Samples at three different initial concentrations were loaded into the Yphantis cell, which was removed from the glovebox and spun at 8000−15 000 rpm at 4−20 °C in an analytical ultracentrifuge (Beckman Optima XLA). Samples were deemed to have reached equilibrium when the 420 nm absorbance pattern afforded therefrom ceased changing with time (after 10−20 h). After centrifugation, samples were removed, and their catalytic activities were determined and compared to an equivalent sample that had remained in the glovebox during the experiment.

RESULTS

Native $CODH_{Ct}$ incubated in SDS and then electrophoresed under nondenaturing conditions dissociated into FM-CODH, α, and other species, as described earlier (Xia & Lindahl, 1995). FM-CODH was isolated in large quantities by slicing the corresponding band from preparative gels and electroeluting it from the polyacrylamide. α was isolated as previously described (Xia et al., 1995). Nondenaturing electrophoretic bands of isolated α, FM-CODH, and native $CODH_{Ct}$ are shown in Figure 1 (lanes A, B, and C, respectively). As predicted from nondenaturing gels of $CODH_{Ct}$ samples incubated in SDS, isolated FM-CODH migrated faster than native enzyme, and isolated α migrated faster than either FM-CODH or native enzyme. Incubating FM-CODH and α in a 1:1 molar ratio yielded a species (the reconstituted enzyme) that migrated at the same rate as native enzyme (Figure 1D). The SDS−PAGE gel of isolated α, FM-CODH, and native $CODH_{Ct}$, shown in Figure 2, reveals that the isolated species were about 90% pure. Densitometry analysis of bands from native $CODH_{Ct}$ and FM-CODH afforded the β/α ratios given in Table 2.

The molecular masses of native $CODH_{Ct}$, FM-CODH, α, and the reconstituted enzyme were determined by analytical ultracentrifugation using the sedimentation equilibrium method (Van Holde, 1985). Sedimentation profiles were monitored at 420 nm. Selected data sets are shown in Figure 3. The

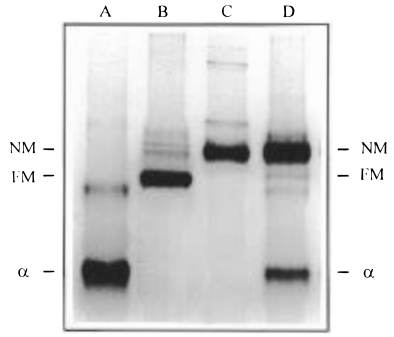

FIGURE 1: Nondenaturing 8% polyacrylamide gel of isolated CODH species. (A) 5 μg of isolated α; (B) 10 μg of isolated FM-CODH (110 units/mg); (C) 5 μg of native $CODH_{Ct}$, batch 2; (D) 10 μg of isolated FM-CODH plus 3.6 μg of isolated α (yielding a 1:1 molar ratio of FM-CODH to α).

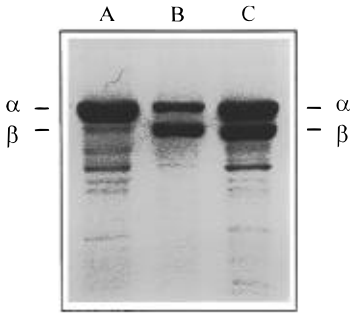

FIGURE 2: SDS−polyacrylamide (8%) gel of isolated CODH species. (A) 4.3 μg of isolated α; (B) 4.3 μg of FM-CODH, 110 units/mg; (C) 4.3 μg of native $CODH_{Ct}$, batch 2.

Table 2: Experimental and Theoretical Molecular Masses and β/α Ratios of $CODH_{Ct}$ and SDS-Induced Dissociation Products Assuming Various Quaternary Structures

species	exptl M_r (ave) (Da)	exptl β/α	struc-ture	theor M_r (Da)	theor β/α	consis-tent?
native	300 000	0.85	$\alpha_1\beta_1$	155 000	1	no
native	300 000	0.85	$\alpha_2\beta_2$	310 000	1	yes
native	300 000	0.85	$\alpha_3\beta_3$	465 000	1	no
FM	210 000	2.2	$\alpha_1\beta_2$	228 000	2	yes
FM	210 000	2.2	$\alpha_1\beta_3$	301 000	3	no
α	90 000	0	α_1	82 000	0	yes
α	90 000	0	α_2	164 000	0	no
recon	280 000	not determined	$\alpha_1\beta_1$	155 000	1	no
recon	280 000	not determined	$\alpha_2\beta_2$	310 000	1	yes
recon	280 000	not determined	$\alpha_3\beta_3$	465 000	1	no

data were fit to eq 1where A_r is the absorbance at 420 nm at

$$\ln A_r = [M_r\omega^2(1 - \bar{\nu}\rho)(r^2 - r_o^2)]/2RT + \ln A_{ro} \quad (1)$$

radius r, M_r is the molecular mass, ω is the angular velocity, $\bar{\nu}$ is the partial specific volume, ρ is the density of the buffer, r_o is the radius corresponding to the first data point, R is the gas constant, T is the absolute temperature, and A_{ro} is the absorbance at 420 nm at r_o. The values of $\bar{\nu}$ used, 0.741 cm^3/g for native $CODH_{Ct}$, FM-CODH, and the reconstituted enzyme, and 0.743 cm^3/g for α, were calculated as described (Cohn & Edsall, 1943). The values of ρ used (1.002−1.011 g/cm^3) were experimentally determined for the individual buffers at the temperature of each experiment. The resulting best-fit M_r values for each experiment are listed in Table 1. The average M_r values and the estimated overall uncertainties for native $CODH_{Ct}$, FM-CODH, α, and the reconstituted enzyme were 300 000 ± 30 000, 210 000 ± 30 000, 90 000 ± 14 000, and 280 000 ± 40 000 Da, respectively. CO oxidation activities of native enzyme, FM-CODH, and the reconstituted species are also listed in Table 1.

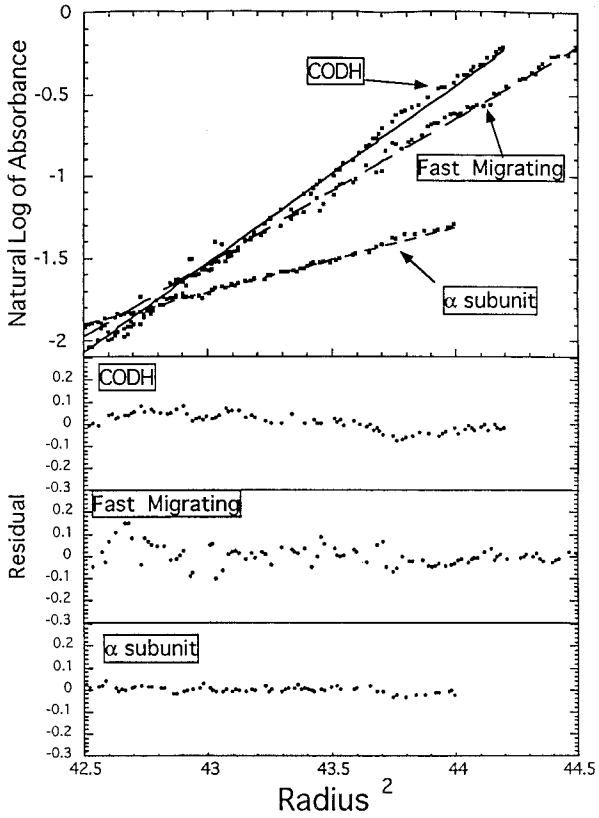

FIGURE 3: Analytical ultracentrifugation of CO dehydrogenase and dissociation products. Top panel: The solid line is the best-fit of the native $CODH_{Ct}$ data from the first native experiment listed in Table 1 to eq 1 (20 °C, 8000 rpm). The long-dashed line is the equivalent fit for the FM-CODH data of the last FM-CODH experiment listed in Table 1 (4 °C, 8000 rpm). The short-dashed line is the equivalent fit for the α subunit data for the last α experiment listed in Table 1 (4 °C, 8000 rpm). Lower three panels: Residuals (simulations minus data) for the native, FM-CODH, and α data sets of panel A, respectively.

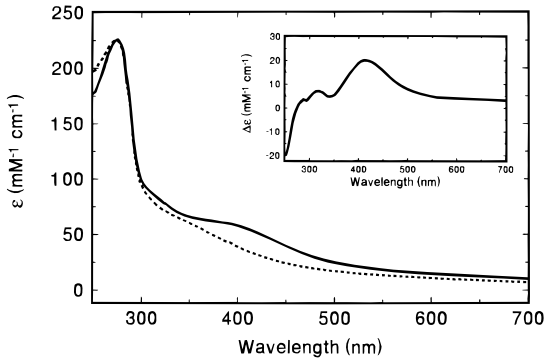

FIGURE 4: Electronic absorption spectra of thionin-oxidized (solid line) and CO-reduced (dashed line) FM-CODH. The sample was 6.1 μM, with 300 units/mg of CO oxidation activity. The inset is the oxidized-minus-reduced difference spectrum.

FM-CODH contained 2.1 ± 0.3 Ni/$\alpha_1\beta_2$, and 16 ± 3 Fe/$\alpha_1\beta_2$ (average ± SD for 7 samples, having specific activities of 310 ± 70 units/mg). The metal content of a sample of native $CODH_{Ct}$, determined coincidentally, was 3.6 Ni and 21.4 Fe/$\alpha_2\beta_2$. Oxidized FM-CODH exhibited a strong shoulder in the 400 nm spectral region (Figure 4, solid line). Such features arise from S → Fe charge-transfer transitions and indicate the presence of Fe-S clusters. The spectral

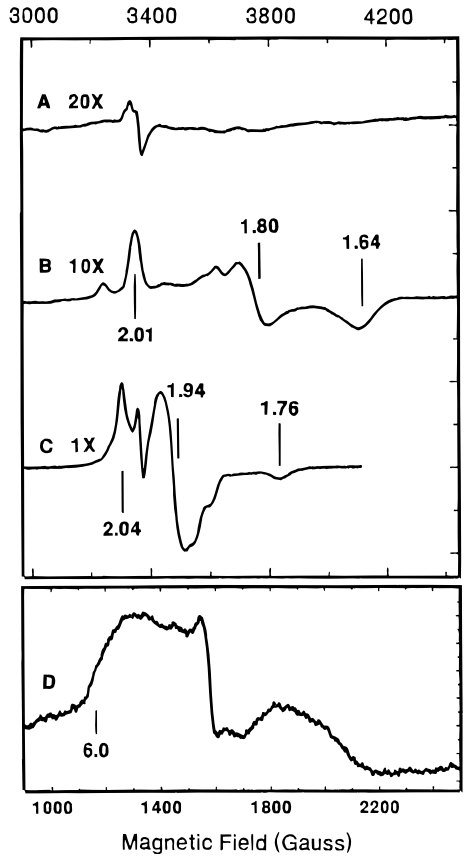

FIGURE 5: EPR of oxidized (A), partially-reduced (B), and fully-reduced (C and D) FM-CODH. For (A), an aliquot of a stock dithionite-free FM-CODH solution (~32 μM; 390 units/mg) was oxidized with a slight molar excess of thionin. For (B), an aliquot was oxidized by *ca*. 0.2 equiv/$\alpha_1\beta_2$ of thionin, and frozen after 10 min incubation. For (C) and (D), an aliquot was reduced by 1 atm of CO. Conditions: microwave frequency, 9.432 GHz; microwave power, 20 mW for (A), (B), and (D), 50 μW for (C); temperature, 10 K. The intensities of (A), (B), and (C) have been normalized for differences in protein concentration and microwave power, and they can be compared directly after the displayed intensities of (A) and (B) are divided by 20 and 10, respectively.

intensity declined when the sample was exposed to CO (Figure 4, dashed line), indicating reduction of the clusters. The difference between the spectra of oxidized and reduced samples (Figure 4, inset) was largest at 420 nm, and corresponded to $\Delta\epsilon_{420} = 20\,000\ M^{-1}\ cm^{-1}$.

Dithionite-free FM-CODH to which 0.2 equiv/mol of thionin was added exhibited an EPR signal (Figure 5B) with $g_{av} = 1.82$ ($g = 2.01$, 1.80, and 1.64). The intensity of this signal quantified to 0.35 ± 0.07 spin/mol (average ± SD for three samples). FM-CODH reduced by CO or dithionite exhibited signals with $g_{av} = 1.94$ ($g = 2.04$, 1.94, 1.89) and $g_{av} = 1.86$ ($g \sim 1.97$, 1.87, 1.75) (Figure 5C). The intensity of both signals together corresponded to 1.8 ± 0.2 spin/mol (average ± SD for three samples). Such samples also exhibited weak low-field features between $g = 4$ and 6, typical of $S = {}^3/_2$ $[Fe_4S_4]^{1+}$ clusters (Figure 5D). Fully-oxidized FM-CODH was nearly devoid of EPR signals (Figure 5A).

DISCUSSION

Quaternary Structure of Native CODH$_{Ct}$. The quaternary structure of an enzyme is among its most fundamental characteristics, and it is unfortunate that the structure of CODH$_{Ct}$ has remained unestablished for over a decade. The original purpose of this investigation was to establish the quaternary structure and metal cluster content of FM-CODH, but unexpected results (indicating $\alpha_1\beta_2$, see below) prompted us to reinvestigate the quaternary structure of native enzyme. The results of the sedimentation equilibrium and SDS−PAGE experiments reported here, when compared to theoretical values assuming various quaternary structures (Table 2), indicate that native CODH$_{Ct}$ is an $\alpha_2\beta_2$ tetramer rather than the $\alpha_3\beta_3$ structure previously proposed (Ragsdale et al., 1983b). The earlier proposal was based on the migration rate of CODH$_{Ct}$ through size-exclusion chromatography columns calibrated with various protein standards. Using such methods, apparent molecular masses of 410 000−460 000 Da were obtained (Drake et al., 1980; Ragsdale et al., 1983b; Ramer et al., 1989). This method of determining molecular mass is unreliable because migration rates are functions of molecular shape as well as mass. Thus, nonspherically-shaped proteins migrate faster than spherically-shaped ones, and yield higher apparent molecular masses. Sedimentation equilibrium profiles, on the other hand, are functions only of molecular mass, angular velocity, temperature, the partial specific volume of the protein, and the density of the buffer. Angular velocity, temperature, and buffer-density are easily determined, and the partial specific volumes of all proteins are remarkably similar, and can be rather accurately estimated. Given that CODH$_{Ct}$ migrates substantially slower than expected for a spherical molecule of the same molecular mass, it probably has a nonspherical shape.

Diekert (1988) first proposed that CODH$_{Ct}$ had a nonspherical $\alpha_2\beta_2$ structure, and in hindsight, this report should have received more recognition that it did. Unfortunately, the forum used to published those results (a chapter of a book), the lack of experimental detail reported, and the attention consumed by another controversy at that time (whether CODH$_{Ct}$ contained a γ subunit) combined to detract from the impact of that study. Our results and conclusions confirm and strengthen Diekert's proposal.

Quaternary Structure of FM-CODH and Stoichiometry of the Dissociation Induced by SDS. The experimentally-determined molecular mass and β/α ratio of FM-CODH indicate an $\alpha_1\beta_2$ quaternary structure (Table 2). Consequently, the SDS-induced dissociation of native CODH$_{Ct}$ appears to involve the loss of a single α subunit, as given in eq 2:

$$\alpha_2\beta_2 \rightleftharpoons \alpha_1\beta_2 + \alpha \qquad (2)$$

Since FM-CODH incubated with isolated α yielded a species that had the molecular mass and migration properties of native enzyme, reaction 2 *appears* to be reversible. However, we have not determined whether the reconstituted species has all the properties of native CODH$_{Ct}$, and so reaction 2 may be reversible only in the sense that isolated α and $\alpha_1\beta_2$ can reassociate. Nevertheless, the ability of these species to reassociate indicates that their dissociated states have tertiary structures similar to those of native enzyme. This, in turn, suggests that the metal centers in isolated α and $\alpha_1\beta_2$ have spectroscopic, redox, and chemical properties that are both similar and relevant to the properties of the centers in native enzyme.

Characterization of FM-CODH. The CO oxidation activities of native CODH$_{Ct}$ and FM-CODH can be compared by

converting specific activities into turnover frequencies. Using $M_r = 310\,000$ Da for native $CODH_{Ct}$ yields turnover frequencies ranging from 1100 to 1300 s^{-1} (at 50 °C). A similar calculation for FM-CODH (using $M_r = 228\,000$ Da) yields values ranging from 20 to 1400 s^{-1}. Thus, the CO oxidation activity of FM-CODH was more variable than native enzyme, but some samples had activities as high as native $CODH_{Ct}$. Samples of FM-CODH with low CO oxidation activities may have been damaged during the extensive isolation procedures (electrophoresis through polyacrylamide gels containing the oxidant ammonium persulfate and the radical initiator TEMED, followed by electroelution) endured *exclusively* by the FM-CODH samples. We suspect that the activities of native $CODH_{Ct}$ samples would have been similarly diminished had they been treated similarly. That some FM-CODH samples had similar activities to native enzyme suggests that loss of an α subunit has no effect on CO oxidation activity and is not required for this activity. We previously argued that the β subunit was responsible for CO oxidation activities and that α was used for the acetyl-CoA synthase activity (Xia & Lindahl, 1995). The matching β content and CO oxidation activities of FM-CODH and native $CODH_{Ct}$ provides further support for this proposal.

The metal contents of native $\alpha_2\beta_2$ (3.5−4 Ni, 22−26 Fe), $\alpha_1\beta_2$ (1.8−2.4 Ni, 13−19 Fe), and isolated α (*ca*. 1 Ni, 4 Fe) indicate that as few as 0 Ni or Fe and no more than 1 Ni and ∼8 Fe per $\alpha_2\beta_2$ are unaccounted for in the SDS-induced dissociation given in eq 2. Given these metal contents and the sizable uncertainties associated with metal determinations in proteins, we estimate that each β subunit contains 0.5−1 Ni and 5−9 Fe. How are these metal ions organized?

The broad absorption in the 400 nm region of the UV−vis spectra of FM-CODH indicates that FM-CODH contains Fe-S clusters. $\Delta\epsilon_{420}$ (20 000 M^{-1} cm^{-1}) is reasonably consistent with that expected, given the $\Delta\epsilon_{420}$ of native $CODH_{Ct}$ (29 400 M^{-1} cm^{-1}; Shin et al., 1992) and isolated α (4000 M^{-1} cm^{-1}), and the stoichiometry of reaction 2. These results suggest little if any loss of Fe-S clusters during the preparation of FM-CODH.

The EPR spectra of FM-CODH provide some indication of the clusters in FM-CODH. The presence of the $g_{av} =$ 1.82 and 1.86 signals indicates that FM-CODH contains the C-cluster. Moreover, the quantified intensity of the $g_{av} =$ 1.82 signal (0.35 spin/$\alpha_1\beta_2$) is quite similar to that obtained for the equivalent signal in native $CODH_{Ct}$ (0.36 ± 0.12 spin/$\alpha_2\beta_2$; Lindahl, 1990a). This suggests that the C-cluster is located in the β subunit, for if it was in α the spin intensity would have been about half of that of native $CODH_{Ct}$. The presence of the $g_{av} = 1.94$ signal in FM-CODH also demonstrates that it contains the B-cluster. The combined spin intensities of the $g_{av} = 1.94$ and 1.86 signals (1.8 ± 0.2 spins/$\alpha_1\beta_2$) are quite similar to that reported for the same two signals in native enzyme (1.9 spins/$\alpha_2\beta_2$; Lindahl et al., 1990a). Again, this suggests that the B-cluster is located in the β subunit. The absence of the NiFeC signal in FM-CODH suggests that the A-cluster is either absent or present in an altered form. Mössbauer studies are underway to examine these assignments further.

Organization of Subunits in $CODH_{Ct}$. Besides $\alpha_1\beta_2$ and α_1, the only other possible products for the dissociation of $\alpha_2\beta_2$ are $\alpha_2\beta_1$, $\alpha_1\beta_1$, α_2, β_2, and β_1. The $\alpha_2\beta_1$ and $\alpha_1\beta_1$ forms do not appear to be stable, as there is no evidence for them in nondenaturing gels of samples incubated with a wide range of SDS concentrations (490−52 000 equiv/$\alpha_2\beta_2$) and incubation temperatures (7−70 °C) (Xia & Lindahl, 1995). A band consisting only of β is observed, but it is somewhat unstable and is largely devoid of metal centers (Xia & Lindahl, 1995). We do not know the quaternary structure of β in this band (i.e., whether it is β_1 or β_2), but the same band is observed under rather extreme conditions (70 °C, 52 000 equiv/$\alpha_2\beta_2$ of SDS) that would probably cause β_2 dimers to dissociate. Thus, we suspect that the observed band consists of β_1 monomers, and that β_2 decomposes into metal-deficient β subunits when the remaining α subunit dissociates from $\alpha_1\beta_2$, as given in eq 3:

$$\alpha_1\beta_2 \rightarrow \alpha + 2\beta + \text{metal ions} \qquad (3)$$

Accordingly, the $\alpha_2\beta_1$ and $\alpha_1\beta_1$ decomposition products may be unstable because they each have only one β subunit.

These characteristics of reactions 2 and 3, the nonspherical shape of $CODH_{Ct}$, the high concentration of metal ions in β relative to α, and the reported electron micrographs of the enzyme provide evidence for how the subunits are organized. Sundaresh et al. (1993) describe the enzyme as having "a central, rhomboidal protein core of about 125 Å in length" with a "near circular protein mass of about 50 Å in diameter... attached to either side of the longitudinal end of this rhomboidal core". They also mentioned that the metals of the enzyme were "concentrated in the central core". Since the metal content of β appears to be roughly twice that of α, the two β subunits probably form this core. Since α subunits can dissociate from native enzyme to yield FM-CODH, these subunits may form the terminal protein masses. Thus, we propose a linear $\alpha\beta\beta\alpha$ structure for $CODH_{Ct}$. Further experimental support is required before this arrangement can be considered established.

Assuming this $\alpha\beta\beta\alpha$ subunit arrangement, the enzyme may decompose by any of three mechanisms: (1) both α subunits might dissociate from the $\beta\beta$ core with the same K_d, resulting in equal amounts of $\alpha\beta\beta$ and $\beta\beta\alpha$; (2) one α subunit may bind the core weaker than the other α does, resulting in either exclusively $\alpha\beta\beta$ or $\beta\beta\alpha$; and (3) both α subunits may bind the core with the same K_d, and the dissociation of either may strengthen the binding of the other. This mechanism would also result in equal amounts of $\alpha\beta\beta$ and $\beta\beta\alpha$. Mechanisms 1 and 3 imply homogeneous α subunits in each tetramer, while 2 implies that the two α subunits in each molecule are different. Mechanism 3 also requires cooperativity between subunits. At this point, none of the mechanisms can be exlcuded. The one implying heterogeneity may appear unlikely, since each α subunit is coded by the same gene, but there is some evidence for heterogeneity (Lindahl et al., 1990a,b; Lu & Ragsdale, 1991; Shin et al., 1993).

Summary. We have shown that $CODH_{Ct}$ has an $\alpha_2\beta_2$ quaternary structure and a nonspherical shape. The enzyme may consist of a β_2 core with terminal α subunits, though further studies are required to establish this. SDS induces the dissociation of native $CODH_{Ct}$ into one α subunit and an $\alpha_1\beta_2$ species. Loss of the remaining α subunit from $\alpha_1\beta_2$ appears to disrupt the β_2 core, leading to metal loss from β. Incubation of $\alpha_1\beta_2$ with α yields an $\alpha_2\beta_2$ species, indicating that the gross structures of the dissociated species are maintained during dissociation. The $\alpha_1\beta_2$ form (called FM-CODH) had full CO oxidation activity and contained *ca*.

2.1 Ni and 16 Fe. Reduced $\alpha_1\beta_2$ exhibited the $g_{av} = 1.82$, 1.94, and 1.86 EPR signals, characteristic of the B- and C-clusters found in native $CODH_{Ct}$. Quantitation of the EPR signal intensities suggests that both clusters are located in the β subunit. The lack of substantial CO/acetyl-CoA exchange activity and NiFeC signal intensity arising from the active site cluster for this activity (the A-cluster) suggests that the A-cluster has been destroyed or altered. Further studies aimed at understanding the distribution of subunits in the enzyme, as well as the distribution of metal centers within each subunit, are underway.

ACKNOWLEDGMENT

We thank David P. Barondeau for generously donating batch 2 of $CODH_{Ct}$ for these studies, Dr. David P. Giedroc for use of the atomic absorption spectrophotometer, and Dr. Ryland F. Young for use of the densitometer. The ultracentrifuge utilized for these studies is a component of the facilities made available by the Center for Macromolecular Design.

NOTE ADDED IN PROOF

Very recently we obtained evidence that the A-cluster is located in the α subunit (Xia & Lindahl, 1996). Given the evidence presented here that the B- and C-clusters are located in the β subunit, and that the subunits are arranged as $\alpha\beta\beta\alpha$, we propose that the clusters and subunits of $CODH_{Ct}$ are arranged as illustrated in Figure 6. The model is probably correct in the outline form presented here, but it may undergo some minor modifications (e.g., the A-cluster in one α subunit may not be spectroscopically equivalent to that in the other α subunit) once the heterogeneity problem described in the text is better understood.

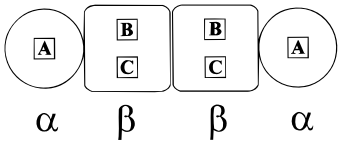

FIGURE 6: Proposed arrangement of clusters and subunits in CODH.

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BI9511853