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Involvement of Tryptophan Residues at the Coenzyme A Binding Site of Carbon Monoxide Dehydrogenase from *Clostridium thermoaceticum*†

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ABSTRACT: Carbon monoxide dehydrogenase (CODH) from *Clostridium thermoaceticum* plays a central role in the newly discovered acetyl-CoA pathway [Wood, H. G., Ragsdale, S. W., & Pezacka, E. (1986) *FEMS Microbiol. Rev. 39*, 345–362]. The enzyme catalyzes the formation of acetyl-CoA from methyl, carbonyl, and CoA groups, and it has specific binding sites for these moieties. In this study, we have determined the role of tryptophans at these subsites. *N*-Bromosuccinimide (NBS) oxidation of the exposed and reactive tryptophans (5 out of a total of ~20) of CODH at pH 5.5 results in the partial inactivation of the exchange reaction (~50%) involving carbon monoxide and the carbonyl group of the acetyl-CoA. Also, about 70% of the acetyl-CoA synthesis was abolished as a result of NBS modification. The presence of CoA (10 μM) produced complete protection against the partial inhibition of the exchange activity and the overall synthesis of acetyl-CoA caused by NBS. Additionally, none of the exposed tryptophans of CODH was modified in the presence of CoA. Ligands such as the methyl or the carbonyl groups did not afford protection against these inactivations or the modification of the exposed tryptophans. A significant fraction of the accessible fluorescence of CODH was shielded in the presence of CoA against acrylamide quenching. On the basis of these observations, it appears that certain tryptophans are involved at or near the CoA binding site of CODH.

Carbon monoxide dehydrogenase (CODH)[1] from *Clostridium thermoaceticum* catalyzes the final steps of the newly proposed acetyl-CoA pathway (Wood et al., 1986a–c). Several of the enzymes involved in this complex pathway have been purified and characterized (Drake et al., 1981; Hu et al., 1984; Ragsdale & Wood, 1985; Pezacka & Wood, 1986). CODH is an $\alpha_3\beta_3$ metalloenzyme and has a molecular weight of 440 000 (Ragsdale et al., 1983a,b). Ragsdale and Wood (1985) have shown that CODH per se catalyzes exchange of ^{12}CO with [1-^{14}C]acetyl-CoA ($CH_3{}^{14}COSCoA + {}^{12}CO \leftrightarrow CH_3{}^{12}COSCoA + {}^{14}CO$). This exchange involves cleavage of both the C–C and C–S bonds of the acetyl-CoA, resynthesis of the acetyl-CoA, and binding of the CH_3, CO, and CoA groups to CODH. On the basis of this exchange reaction, they have proposed that CODH catalyzes the final steps of the

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[1] Abbreviations: CODH, carbon monoxide dehydrogenase; DTT, dithiothreitol; NBS, *N*-bromosuccinimide; CoA, coenzyme A; Trp, tryptophan; ESR, electron spin resonance; CD, circular dichroism; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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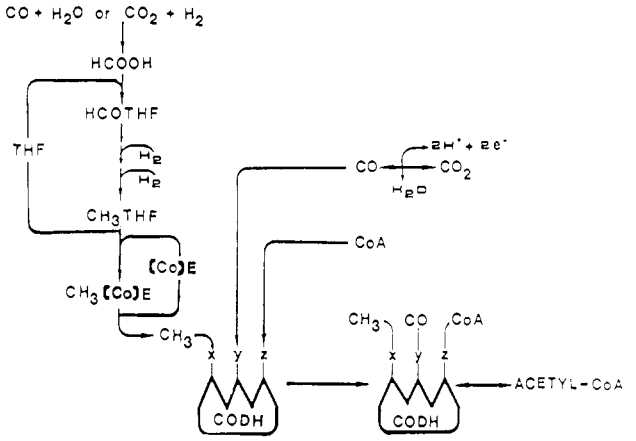

FIGURE 1: Abbreviated scheme of acetyl-CoA pathway of heterotrophic and autotrophic growth. CO or CO_2 is converted via formate and THF to CH_3THF which is the source via the [Co]E and CODH of the methyl group of acetyl-CoA. The CO and CoA are added to CODH, which then catalyzes the synthesis of acetyl-CoA. THF is tetrahydrofolate, [Co]E is corrinoid enzyme, and carbon monoxide dehydrogenase is indicated with three subsites x, y, and z for the methyl, the carbonyl, and the CoA groups, respectively.

synthesis of acetyl-CoA as illustrated in Figure 1. From ESR studies, it has been proposed that the Ni–Fe center is involved during interaction of the carbonyl group with CODH (Ragsdale et al., 1985). Recently, Pezacka and Wood (1988) have shown using either methyl iodide or methyl corrinoid enzyme that the methyl group is bound to a cysteine residue of CODH. With this methylated CODH, CO, and CoA, acetyl-CoA is formed without a requirement for other enzymes, thus providing direct evidence of the role of CODH in the synthesis of acetyl-CoA as shown in Figure 1. Arginine residues have been proposed to be involved at the CoA binding site of the enzyme (Ragsdale & Wood, 1985).

In this paper, using intrinsic fluorescence, *N*-bromosuccinimide (NBS) modification, and solute quenching of the Trp residues of CODH, we have examined the role of Trp residues in the various ligand binding sites of the enzyme. The results indicate that Trp residues are involved at the CoA binding site of CODH.

Materials and Methods

Materials

Acrylamide (electrophoretic grade) was obtained from Accurate Chemical and Scientific Co. CoA and acetyl-CoA were from Sigma. Methyl iodide was from Aldrich.

Methods

CODH was purified from *C. thermoaceticum* and assayed as described by Ragsdale and Wood (1985). The exchange reaction between the carbon monoxide and the carbonyl group of the acetyl-CoA and the overall synthesis of acetyl-CoA were monitored as described earlier (Ragsdale & Wood, 1985; Hu et al., 1984).

Fluorescence Measurements. For fluorescence studies, the enzyme was prepared anaerobically by transferring suitable aliquots of the stock enzyme solution using a gas-tight syringe into an anaerobic cell containing appropriate volumes of the buffer. A degassed 50 mM potassium phosphate buffer, pH 6.0, containing 4 mM DTT was used for dilution. Fluorescence emission spectra were recorded with an Aminco–Bowman spectrofluorometer by scanning from 300 to 400 nm with the spectrometer in the ratio mode. Fluorescence measurements were made in the range where fluorescence emission was linear with protein concentration, and the absorbance of all protein solutions employed was between 0.05 and 0.1 at the excitation wavelength.

The relative quantum yields (q_{prot}) were calculated by comparing the absorbance (Abs) at 295 nm and the areas (A) of the emission spectra of the protein with a standard of known quantum yield (q_{st}) according to eq 1. The standard was a

$$q_{prot} = q_{st}A_{prot}Abs_{st}/A_{st}Abs_{prot} \quad (1)$$

crystallized preparation of *N*-acetyltryptophanamide. A quantum yield of 0.14 has been reported for this compound in aqueous solution (Eisinger & Navon, 1969).

N-Bromosuccinimide Oxidation of CODH. The procedure employed for NBS modification was adapted from the methods of Patchornik et al. (1960) and Spande and Witkop (1967). Appropriate enzyme solutions (1–2 mg/mL) were prepared in 100 mM sodium acetate solution, pH 5.5, containing 4 mM DTT. A stock solution of NBS (10 mM) was used for the modification studies. Aliquots of NBS (5–10 μL) were mixed with CODH (1 mL) in a tightly sealed quartz cuvette with a 10-mm path length. Following 2–3 min of incubation after each NBS addition, the absorbance at 280 nm was recorded and corrected for dilution. The number of modified Trp residues was calculated as described by Spande and Witkop (1967). In addition, appropriate aliquots were withdrawn and diluted with a suitable concentration of *N*-acetyltryptophanamide to quench any residual NBS. The activities of the modified enzymes in the exchange and the synthesis of acetyl-CoA were determined and expressed as a percentage of the activities of the unmodified enzyme. The chemical modification of the enzyme with NBS was also monitored by following the changes in fluorescence intensity at the emission maximum (339 nm) with the excitation wavelength fixed at 295 nm.

In another series of parallel experiments, CODH solutions were treated with NBS in the presence of 10 μM each of the following: CoA and acetyl-CoA. The extent of inactivation and number of modified Trp residues were determined as described above.

Acrylamide Quenching Studies. Fluorescence intensities were determined by using an excitation wavelength of 295 nm and by continuous monitoring of the emission at 339 nm. The final intensity at any given acrylamide concentration was taken as a time-averaged value (n = 10). Additions of acrylamide were made from a concentrated (8.0 M) stock solution. Fluorescence was corrected for dilution and inner filter effects (Hélène et al., 1969). A correction for absorption of the acrylamide itself at 295 nm was made according to McClure and Edelman (1967) using a molar extinction coefficient of 0.29 M^{-1} cm^{-1} at 295 nm.

Data Analysis. Fluorescence quenching was analyzed according to the modified Stern–Volmer relationship (eq 2) (Lehrer, 1971). From a plot of $F_0/(F_0 - F)$ versus $[Q]^{-1}$, the

$$F_0/(F_0 - F) = 1/f_{a(eff)} + 1/f_{a(eff)}K_{Q(eff)}[Q] \quad (2)$$

values of $f_{a(eff)}$, the maximum fractional accessible fluorescence (1/intercept), and $K_{Q(eff)}$, the effective collisional quenching constant (intercept/slope), are obtained.

Circular Dichroism Studies. The circular dichroism (CD) spectra were recorded with a Jasco Model J-10 spectropolarimeter (Jasco, Inc., Easton, MD), calibrated with *d*-10-camphorsulfonic acid (Cassim & Young, 1969). Samples were placed in an anaerobic cylindrical quartz cell with a path length of 1 mm. The mean residue ellipticity, $[\theta]$, values were calculated from eq 3 (Adler et al., 1973) where H is the height

$$[\theta] = HS(MRW)/Cl \quad (3)$$

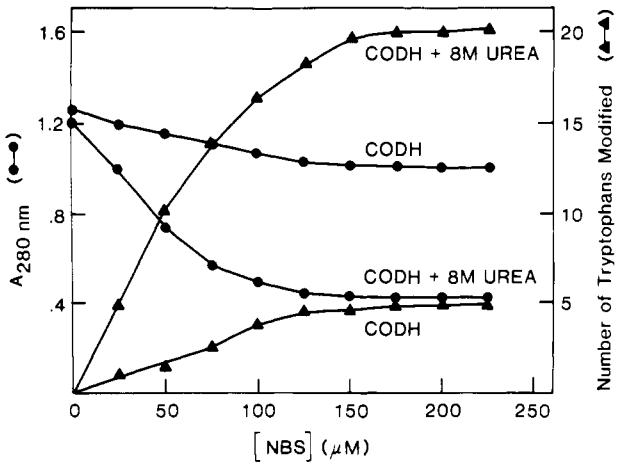

FIGURE 2: Oxidation of the tryptophans (▲) and the absorbance at 280 nm (●) of CODH with varied concentrations of NBS. The modifications were done with 2 μM CODH at pH 5.5 in the presence and absence of 8 M urea.

Table I: NBS Modification of Tryptophan Residues and Fluorescence Parameters of CODH[a]

sample	no. of Trp modified	fluorescence quantum yield	λ_{max} (nm)	acrylamide quenching[b] $K_{Q(eff)}$ (M^{-1})	acrylamide quenching[b] $f_{a(eff)}$
CODH	5	0.028	339	8.15	0.79
CODH + CoA[c]	0	0.025	337	6.31	0.47
CODH + 8 M urea	19–20	0.144	360	10.12	0.99
CODH + CH_3I and CO[d]	5	0.028	339	ND[e]	ND

[a] The following concentrations of CODH were used: for NBS modification, 2 μM in 100 mM sodium acetate, pH 5.5, containing 4 mM DTT; for fluorescence and acrylamide quenching, 0.23 μM in 50 mM potassium phosphate buffer, pH 6.0, containing 4 mM DTT. [b] The effective collisional quenching constant [$K_{Q(eff)}$] and $f_{a(eff)}$ were determined by using eq 2, as described under Methods. [c] The concentration of CoA was 10 μM, and a similar trend was observed with acetyl-CoA (10 μM). [d] The concentration of CH_3I was 2 μM, and the medium was saturated with CO. [e] ND = not determined. The number of tryptophans modified is calculated on the basis of the $\alpha_3\beta_3$ form of CODH.

of the peak, S is the sensitivity, MRW is the mean residue weight (~115), C is the concentration of the protein in grams per liter, and l is the path length of the cell. The protein content was estimated by the Rose Bengal assay (Elliot & Brewer, 1978). The relative proportions of α-helix, β-sheet, and random-coil structures were estimated by the method of Siegel et al. (1980).

RESULTS

Determination of Total and Exposed Tryptophans of CODH. The total tryptophans of CODH were determined by titration with NBS of the enzyme denatured in 8 M urea. From analyses of several enzyme preparations, the Trp content was found to vary between 19 and 20 (Figure 2 and Table I). In the absence of urea, consistently about five tryptophans were modified at pH 5.5 (Figure 2). The remaining tryptophans are less reactive or present in the interior of the protein structure or located at the subunit interface and thus not available for NBS reaction.

Determination of the Effect of Modification of Tryptophans of CODH on Its Activity both in the Presence and in the Absence of Substrates. NBS inhibited the exchange reaction between carbon monoxide and the carbonyl group of acetyl-CoA (Figure 3). A maximum of 50% of the exchange activity is lost with the addition of 150 μM NBS (final concentration).

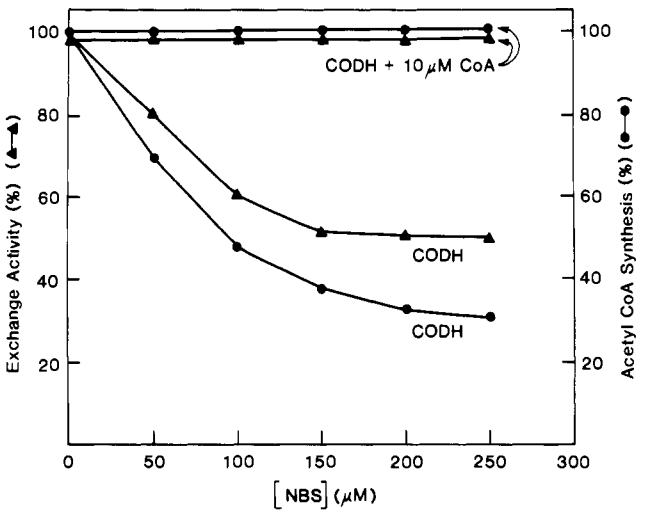

FIGURE 3: Effect of varied concentrations of NBS on the exchange activity catalyzed by CODH (▲) and the synthesis of acetyl-CoA from CO, CoA, and CH_3THF (●). CODH (2 μM) was incubated with indicated concentrations of NBS for 2–3 min in 100 mM sodium acetate, pH 5.5, containing 4 mM DTT. Suitable aliquots were used for the measurements of the residual activities.

On the other hand, about 70% of the synthesis of acetyl-CoA is abolished in the presence of about 200 μM NBS. Between 150 and 200 μM NBS, about five tryptophans of CODH are modified (Table I and Figure 2). The observed inactivation of CODH in the presence of NBS might be due to modification of tryptophans critical for activity at any one of the three binding sites involving the methyl, the carbonyl, and the CoA groups. Previously, it has been shown that methyl iodide can serve as a methyl donor for the synthesis of acetyl-CoA (Pezacka & Wood, 1988; Shanmugasundaram et al., 1988). NBS modification of the enzyme, therefore, was carried out in the presence of methyl iodide, carbon monoxide, or CoA, and the number of tryptophans modified was determined. As shown in Table I, in the presence of both methyl iodide and carbon monoxide, about five tryptophans were modified whereas in the presence of 10 μM CoA at pH 5.5, none of the tryptophans was modified. Thus, only CoA provided protection against modification of tryptophans of the intact enzyme, suggesting that certain tryptophans are in close proximity to or at the CoA binding site of CODH. Also, in the presence of 10 μM CoA, there was no inhibition by NBS of the exchange activity or of the synthesis of acetyl-CoA (Figure 3). Similar protection against inhibition was not observed in the presence of either CO or methyl iodide (data not shown), suggesting the absence of Trp residues at the binding sites of these functional groups.

Demonstration That Extensive Conformational Change Has Not Occurred during NBS Modification of CODH. The observed loss of activity with NBS oxidation of the Trp residues of the intact enzyme may be due to either modification of the essential tryptophans or as a result of conformational change in the enzyme. The fluorescence properties (Figure 4A and Table I) and the CD spectra (Figure 4B) of the native enzyme were compared to those of the enzyme modified with 150 μM NBS. The CD spectra of the native and the NBS-modified enzymes were essentially the same (Figure 4B). By analysis of the CD spectra, it has been shown that CODH is comprised of about 55% α-helix, about 42% β-structure, and the remaining as random-coil structure. The fluorescence of the enzyme as a result of NBS modification was quenched by ~32% without any significant change in the emission maximum (Figure 4A). However, in the presence of 8 M urea, a 4.4-fold increase in the quantum yield of CODH was observed

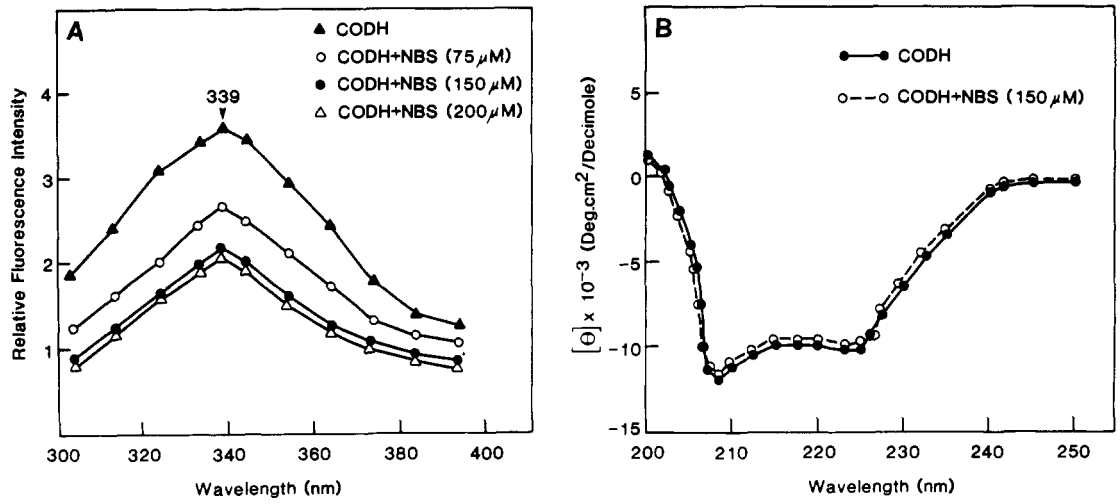

FIGURE 4: Fluorescence emission spectra (A) and circular dichroism (CD) spectra (B) of native and NBS-modified CODH. Dilute solutions of CODH, 0.23 μM for the fluorescence and 0.46 μM for the CD analyses, were used in 100 mM sodium acetate, pH 5.5, containing 4 mM DTT. The other details are as described under Methods.

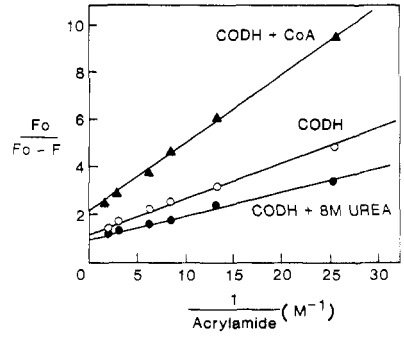

FIGURE 5: Modified Stern–Volmer plots of the fluorescence quenching with acrylamide; 0.23 μM CODH alone (○); in the presence of 10 μM CoA (▲); and in the presence of 8 M urea (●). The enzyme (0.23 μM) was in 50 mM potassium phosphate, pH 6.0, containing 4 mM DTT.

(Table I). Also, there was about a 21-nm red shift in the emission maximum (Table I). On the basis of these observations, it is suggested that the NBS modification of tryptophans has altered only the microenvironment around the CoA binding site without affecting the overall structure of CODH.

Assessment of the Microenvironment of Tryptophans of the CODH both in the Presence and in the Absence of CoA. Acrylamide quenching studies were performed anaerobically with the intact enzyme both in the presence and in the absence of CoA, and the modified Stern–Volmer plot of the quenching data is shown in Figure 5 and Table I. With the intact enzyme, the maximum accessible fluorescence [$f_{a(eff)}$] is 0.79 (Table I), suggesting a fraction of the Trp is not accessible for acrylamide quenching. With the enzyme denatured in 8 M urea, a $f_{a(eff)}$ value of 0.99 was observed. In the presence of 10 μM CoA, the $f_{a(eff)}$ is reduced by about 40%, and the effective collisional quenching constant [$K_{Q(eff)}$] is also reduced significantly as compared to the untreated enzyme (Table I). On the basis of the reduced quenchable fluorescence of the enzyme due to shielding of the tryptophans in the presence of CoA, it is proposed that certain tryptophans are involved at or near the CoA binding site of CODH.

Discussion

In this study, we have examined if tryptophans are involved in the binding sites for the methyl, the carbonyl, and the CoA groups of CODH which catalyzes the synthesis of acetyl-CoA (Ragsdale & Wood, 1985; Wood et al., 1986a–c). Our findings suggest that certain tryptophans are involved at the CoA binding site of CODH.

There are about 20 tryptophans (Figure 2 and Table I) in the intact enzyme which has a subunit stoichiometry of $\alpha_3\beta_3$ and has a molecular weight of 440 000 (Ragsdale et al., 1983a). In the native state, about five tryptophans were modified by NBS. These tryptophans may be considered to be reactive, exposed residues (Figure 2 and Table I). Modification of these tryptophans caused inhibition of (i) the exchange activity between the carbon monoxide and the carbonyl of the acetyl-CoA (~50%) and (ii) the synthesis of acetyl-CoA (~70%) (Figure 3). Both of these inactivations were completely prevented by the addition of CoA to CODH prior to NBS modification. The other ligands such as CH_3I and CO did not protect the partial inactivation caused by NBS (data not shown). Also, the intrinsic fluorescence of the enzyme was quenched in the presence of CoA (Shanmugasundaram et al., 1988) whereas no significant change in the fluorescence was observed when the enzyme was titrated with either methyl iodide (after correcting the effect due to iodide quenching) or carbon monoxide (data not shown). Furthermore, there was no modification of tryptophans of the enzyme when CoA was present in the reaction medium (Table I). NBS oxidation of CODH appears to be specific for tryptophans. This view is supported by the linear decrease in the absorbance of CODH at 280 nm (Figure 2) with increasing concentrations of NBS (up to 125 μM) (Spande & Witkop, 1967). The possible modification of other residues such as tyrosine, histidine, and methionine which have reduced reactivity toward NBS (Schmir & Cohen, 1961; Schechter et al., 1975; Ybarra et al., 1986) was also considered. A comparison of the amino acid compositions of the unmodified and NBS (150 μM) modified CODH's indicated that the contents of tyrosine, histidine, and methionine were not altered (data not shown). On the basis of the above considerations, it is proposed that certain tryptophans are involved at the CoA binding site of CODH.

The partial inhibition of the exchange reaction and of acetyl-CoA synthesis by NBS modification of CODH is not due to gross structural alteration of the enzyme. This view is supported by the observation that there are no significant changes in the CD spectra (Figure 4B) and by the absence of any shift in the emission maximum of the fluorescence spectra of the modified enzyme (Figure 4A). However, the observed quenching of the intrinsic fluorescence (a maximum of 32%) of CODH in the presence of NBS suggests that the

microenvironment of the tryptophans at the CoA site is significantly perturbed.

Additional evidence for the involvement of tryptophans at the CoA binding site has come from acrylamide quenching studies of the enzyme carried out both in the presence and in the absence of CoA. A significant fraction of the accessible fluorescence of the enzyme was shielded in the presence of CoA against acrylamide quenching (Table I), suggesting that certain tryptophans are located at or near the CoA binding site.

Previously Ragsdale et al. (1985) have shown that the addition of either CoA or acetyl-CoA to CODH produced a marked change in the ESR signals arising from the Ni–Fe center. On the basis of this observation, they have proposed that the CO and the CoA binding sites are located close to each other. Recently, it has been found that NBS modification of the exposed tryptophans also produced changes in the magnetic properties of the Ni–Fe–C center (Shanmugasundaram et al., 1988). On the basis of the results presented here and other studies (Ragsdale et al., 1985; Shanmugasundaram et al., 1988), it appears that CoA binds to CODH at a site involving exposed tryptophans which are located at or near the Ni–Fe–C center. Tryptophan residues are shown to be present at the subunit contact region close to the CoA binding site of succinyl-CoA synthetase (Prasad et al., 1983). However, in contrast to our results, subsequent NBS modification studies of the synthetase in the presence of CoA analogue (Ybarra et al., 1986) have shown that the tryptophan residue essential for the catalytic activity is not present at the CoA binding site. It will be of great interest to determine if the CoA site of CODH is located at the subunit interface or within a specific subunit of CODH. Studies involving labeling of the enzyme with 2,4-dinitrophenylsulfenyl chloride, a Trp-specific reagent (Scoffone et al., 1968), both in the presence and in the absence of CoA and subsequent SDS–PAGE analysis of the labeled enzyme will be required for further characterization of the CoA binding site of CODH.

Registry No. CODH, 64972-88-9; Trp, 73-22-3; CoA, 85-61-0.

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