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Isolation of a Protein Containing Tightly Bound 5-Methoxybenzimidazolylcobamide (Factor IIIm) from *Clostridium thermoaceticum*†

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ABSTRACT: A corrinoid–protein has been purified from *Clostridium thermoaceticum*. It has a mol wt of about 27,000 and contains 1 mol of tightly bound 5-methoxybenzimidazolylcobamide/mol of protein. The protein is likely a single polypeptide chain but also exists as a dimer of mol wt 55,000. Spectrophotometric and electron paramagnetic resonance (epr) studies indicate that in the native protein the corrinoid exists in a reduced form corresponding to cob(II)alamin that is stable to oxygen. After precipitation with ammonium sulfate the corrinoid–protein is oxidized by air to the cob(III)alamin form. The oxidized corrinoid–protein reacts with cyanide at alkaline pH to form the monocyano derivative Under strong acid conditions the corrinoid–protein does not undergo a spectral change indicating a split of the coordinate bond between the base and the cobalt atom as does the free 5-methoxybenzimidazolylcobamide. The oxidized corrinoid–protein is reduced by extracts of *C. thermoaceticum* with pyruvate as a source of electrons. It is suggested that the corrinoid–protein functions in the synthesis of acetate from 5-methyltetrahydrofolate, which is catalyzed by extracts from *C. thermoaceticum* in the presence of pyruvate.

Acetate is synthesized from CO_2 by *Clostridium thermoaceticum* and it has been shown that methylcorrinoids are intermediates in this synthesis (Ljungdahl *et al.*, 1965; Poston *et al.*, 1966). Thus, in pulse labeling experiments with $^{14}CO_2$, (5-methoxybenzimidazolyl)-*Co*-methylcobamide (methyl factor IIIm) and *Co*-methylcobyric acid are formed labeled with ^{14}C exclusively in their *Co*-methyl groups. When these compounds or [$^{14}CH_3$]cobalamin is incubated with extracts from *C. thermoaceticum* in the presence of pyruvate, acetic acid is formed containing label in the methyl group (Ljungdahl *et al.*, 1965). The methyl group of 5-methyltetrahydrofolate is also labeled in pulse experiments with $^{14}CO_2$ (Parker *er al.*, 1971) and is converted to acetate (Ghambeer *et al.*, 1971). It has been postulated that a protein-bound corrinoid serves as carrier of the methyl group between 5-methyltetrahydrofolate and acetate (Ghambeer *et al.*, 1971). In this paper we wish to report the isolation and some properties of a protein containing tightly bound 5-methoxybenzimidazolylcobamide. Although a role for this protein has not been established in the synthesis of acetate from CO_2 we would like to suggest that it functions in this synthesis.

Experimental Section

Bacterial Culture. C. thermoaceticum was grown in a medium of the following composition in grams per liter: glucose, 18; yeast extract, 5; tryptone, 5; $(NH_4)_2SO_4$, 1; $MgSO_4 \cdot 7H_2O$, 0.25; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.392; $Na_2MoO_4 \cdot 2H_2O$, 0.024; $Co(NO_3)_2 \cdot 6H_2O$, 0.029; $MnCl_2$, 0.0013; Na_2SeO_3, 0.0017; $NaHCO_3$, 16.8; K_2HPO_4, 7; KH_2PO_4, 5.5; sodium thioglycolate, 0.5. This medium differs from that used by Ghambeer *et al.* (1971) by the addition of ferrous ammonium sulfate, manganous chloride, sodium molybdate, and sodium selenite. Andreesen *et al.* (1973) have found that addition of these compounds greatly increases the growth yield. The organism was grown for 48 hr at 55° under CO_2 in carboys containing 18 l. of medium. The cells were harvested in a Sharples centrifuge at room temperature and were stored frozen at −20°.

Materials. Diethylaminoethylcellulose (DE-23) was obtained from Whatman. Before use it was washed with 0.5 M HCl, water, 0.5 M NaOH, and water, and after the pH had been adjusted to 7 by the addition of 1 M KH_2PO_4 it was washed with 0.005 M potassium phosphate, pH 7.0. DEAE-cellulose from Schleicher and Schuell was used in the later steps of the purification of the corrinoid–protein. It was found that the corrinoid–protein eluted more quantitatively from the DEAE-cellulose from Schleicher and Schuell. Aluminum oxide, 0537, was purchased from J. T. Baker. It was soaked with 12 N hydrochloric acid overnight and washed with water; the pH was then adjusted to 7.6 by the addition of 1 N

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potassium hydroxide. It was then left standing for 24 hr and the pH was again adjusted to 7.6, if needed, before it was washed with 0.1 M Tris, pH 7.6. Sephadex G-25, G-50, G-75, and G-100 were obtained from Pharmacia. Ammonium sulfate, special enzyme grade, was obtained from Schwartz Mann and Tris was Trizma Base, reagent grade from Sigma.

Methods. Spectra were obtained with a Beckman Acta V spectrophotometer. Amino acid analyses were performed with a Beckman Model 120 C automatic amino acid analyzer on samples hydrolyzed *in vacuo* with 6 N hydrochloric acid for 24, 48, and 72 hr. Cystine and cysteine were determined as cysteic acid after oxidation with performic acid prior to acid hydrolysis according to Hirs (1967). Tryptophan was determined after acid hydrolysis in the presence of thioglycolic acid according to Matsubara and Sasaki (1969). Sedimentation equilibrium centrifugations were performed according to Schachman (1957) with a Spinco Model E ultracentrifuge fitted for schlieren and interference optics and equipped with a monochromator–scanner. Analytical disc gel electrophoresis was performed according to Brewer and Ashworth (1969) and electrophoresis in the presence of sodium dodecyl sulfate and mercaptoethanol was according to Weber and Osborn (1969). Protein was assayed with the biuret method (Gornall *et al.*, 1949) or by precipitation with 4% (w/v) trichloroacetic acid. In the latter method the turbidity was determined in a spectrophotometer at 660 nm. Bovine serum albumin was used as a standard. Corrinoids were assayed by heating samples at pH 10 for 5 min at 90° with potassium cyanide. The samples were centrifuged if cloudy and the absorption was determined at 580 nm at which wavelength dicyanocorrinoids absorb with ϵ 10.13 $\times$ 10^3 M^{-1} cm^{-1} (Friedrich, 1964).

Purification of 5-Methoxybenzimidazolylcobamide Protein from C. thermoaceticum. Except as noted, Tris-HCl buffer, pH 7.6, was used throughout the purification; a summary is given in Table I.

STEP I. PREPARATION OF EXTRACT. A suspension of 910 g of frozen cells in 1000 ml of 0.05 M potassium phosphate, pH 7.0, containing 0.05 M mercaptoethanol was passed through a Gaulin homogenizer (Manton-Gaulin Mfg. Co., Everett, Mass.). The suspension was centrifuged for 40 min at 27,300*g*. The debris was resuspended in 1000 ml of the phosphate and again passed through the homogenizer. After centrifugation the supernatants containing 93.5 g of protein were combined giving a total volume of 3000 ml, which included some buffer used for wash. The pH, which was 5.9, was adjusted to 6.8 by the addition of 1 M K_2HPO_4.

STEP II. BATCH ABSORPTION WITH DEAE-CELLULOSE. The DEAE-cellulose used in this step was from Whatman. It was equilibrated with 0.005 M potassium phosphate, pH 7.0. Immediately before use excess buffer was removed by filtration. About 1000 ml of the wet filter cake was added to the solution from step I. The suspension was stirred for 30 min and then the DEAE-cellulose containing the corrinoid–protein was filtered off and washed with 2 vol of water. To the filtrate (6200 ml) containing the wash water, 1000 ml of fresh DEAE-cellulose was added and, after stirring for 30 min, the DEAE-cellulose was recovered by filtration and washed with 2 vol of water. The filtrate was saved and used as a source of formyltetrahydrofolate synthetase (Ljungdahl *et al.*, 1970) and methylenetetrahydrofolate dehydrogenase (O'Brien *et al.*, 1973).

The corrinoid–protein and other acid proteins were eluted from the combined batches of the DEAE-cellulose, which were placed on a Büchner funnel, by slowly percolating 3700 ml of 1 M Tris through the DEAE-cellulose cake. The eluate, which was dark brown, contained 33 g of protein.

TABLE I: Purification of Corrinoid (Factor IIIm)–Protein from 910 g of Frozen Cells of *C. thermoaceticum*.

	Step	Total Protein (mg)	Corrinoid (μmol)	Protein: Corrinoid (mg/μmol)
I	Cell-free extract	93,500		
II	DEAE batch absorption	33,000		
III + IV	DEAE chromatography	2,860	17.6	163
V	Aluminum oxide chromatography		16.7	
VI	Sephadex G-100 and G-75	1,135	10.7	106
VII	DEAE gradient elution		10.2	
VIII	Sephadex G-100	153	5.12	29

STEP III. CHROMATOGRAPHY ON DEAE-CELLULOSE. DEAE-cellulose from Schleicher and Schuell was used in this and subsequent steps. It was equilibrated with 1 M Tris pH 7.6 and then washed extensively with water. The 1 M Tris eluate from step II was dialyzed against 4 $\times$ 20 l. of water. A white precipitate was removed by centrifugation, and the solution, 4915 ml, was applied to a DEAE-cellulose column (6 $\times$ 32 cm). The corrinoid–protein was adsorbed together with other colored proteins and the colorless solution, which passed through the column, was discarded. The column was developed with Tris buffer, pH 7.6, in stepwise increasing concentrations. First 1300 ml of 0.01 M Tris was passed through the column, followed by 1300 ml of 0.05 M and 550 ml of 0.2 M. The eluate was colorless and was discarded. Four distinctly colored bands, brown at the top, then red, yellow, and red at the bottom, were then observed on the column. The yellow band and the bottom red band were eluted together by passing 1500 ml of 0.25 M, 820 ml of 0.3 M, and finally 500 ml of 0.325 M Tris through the column. As judged from spectra taken at intervals during the elution, the red band may consist of rubredoxin and the yellow band of flavine. This fraction, 2800 ml, was set aside for future investigation.

The second red band, containing the corrinoid–protein, was next eluted with 500 ml of 0.350 M, 500 ml of 0.375 M, 500 ml of 0.400 M, and finally 1000 ml of 0.425 M Tris. This fraction, 2550 ml, showed distinct absorption peaks at 540, 475, and 355 nm. Finally, the brown band containing ferredoxin (Poston and Stadtman, 1967) was eluted with 700 ml of 1 M Tris. This fraction was also set aside for future investigation.

STEP IV. RECHROMATOGRAPHY ON THE DEAE-CELLULOSE COLUMN OF STEP III. The DEAE-cellulose column of step III was washed with 8 l. of water. The corrinoid–protein solution (2550 ml) from step III was diluted to 6000 ml and applied to the column. The corrinoid–protein was adsorbed on the column. It was washed with 2000 ml of 0.3 M Tris, during which a yellow-brown material was eluted. It was combined with the flavine–rubredoxin fraction from step III. The corrinoid–protein was then eluted with 0.6 M Tris and was ob-

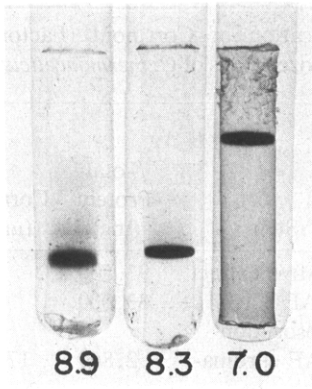

FIGURE 1: Analytical disc and polyacrylamide gel electrophoresis of the corrinoid–protein of step VIII as isolated from *C. thermoaceticum*. About 75 μg of protein was applied to each gel containing 7% acrylamide. The gels were run at pH 7.0, 8.3, and 8.9 in Tris-glycine buffer for 90 min.

tained in a volume of 335 ml containing 2860 mg of protein and 17.6 μmol of cobamide.

STEP V. CHROMATOGRAPHY ON ALUMINUM OXIDE. The corrinoid–protein solution from step IV was applied directly to an aluminum oxide column (4 × 18 cm). The corrinoid–protein was adsorbed and the column was washed with 0.1 M Tris until a yellow material was completely removed. The corrinoid–protein was then eluted with 0.2 M potassium phosphate, pH 7.6, in a volume of 200 ml. The solution was concentrated by using an Amicon Diaflo filter membrane PM 30, to a volume of 44 ml. The cobamide content of this solution was 16.7 μmol.

STEP VI. CHROMATOGRAPHY USING SEPHADEX G-100 AND G-75. Two columns, 5 × 95 cm, containing Sephadex G-100 and Sephadex G-75, respectively, were connected in tandem and equilibrated with 0.05 M Tris. The corrinoid–protein solution, 44 ml, from step V was divided into two equal parts, which were separately run using ascending flow through the Sephadex columns. Two red fractions were obtained, both containing corrinoids. The fraction which eluted first and thus of the highest molecular weight was obtained in a total volume (from both runs) of 1100 ml. It contained 1100 mg of protein and 3.9 μmol of cobamide. As will be shown later this fraction contains a dimer of the corrinoid–protein.

The slower moving low molecular material contained the main part of the corrinoid–protein. It was obtained in a total volume of 700 ml containing 1135 mg of protein and 10.7 μmol of cobamide.

STEP VII. CHROMATOGRAPHY ON DEAE-CELLULOSE USING GRADIENT ELUTION. A DEAE-cellulose column, 4 × 50 cm, was equilibrated with 0.1 M Tris. The low molecular weight corrinoid–protein fraction from step VI was applied to the column, which was then washed with 200 ml 0.2 M Tris. The corrinoid–protein was eluted using a gradient of 500 ml of 0.2 M and 500 ml of 0.6 M Tris. Fractions of 10 ml were collected and the corrinoid–protein was obtained in fractions 5–40, which were combined. The total volume was 365 ml and 10.2 μmol of cobamide was recovered.

STEP VIII. CHROMATOGRAPHY ON SEPHADEX G-100. The corrinoid–protein from step VII was concentrated by precipitation with ammonium sulfate, 80% saturation. The precipitate which was recovered by centrifugation was divided up into two equal aliquots, which were separately run through a Sephadex G-100 column, 2.5 × 95 cm, with 0.1 M Tris as solvent. Fractions of 3.25 ml were collected. Most of the corrinoid–protein was found in fractions 93–100. However, a small amount of the corrinoid–protein eluted in fractions 65–81. The latter fractions were combined and the protein was concentrated by ammonium sulfate precipitation and redissolved in 3.5 ml of 0.1 M Tris. This solution was rechromatographed on the Sephadex G-100 column and the main part of the corrinoid was eluted in fractions 93–100, but again a small amount was obtained in fractions 69–80.

The main corrinoid–protein fractions (93–100) from the three columns were combined. The total volume was 65 ml and it contained 153 mg of protein and 5.12 μmol of 5-methoxybenzimidazolylcobamide.

Purification of the High Molecular Weight Corrinoid–Protein from Step VI and Identification of It as a Dimer of the Corrinoid–Protein of Step VIII. The high molecular weight corrinoid–protein of step VI was purified by gradient chromatography on DEAE-cellulose as described in step VII. The corrinoid–protein was concentrated by ammonium sulfate precipitation and dissolved in 2 ml of 0.1 M Tris. This solution was applied to the Sephadex G-100 column of step VIII. The corrinoid–protein divided up into two fractions. The main part was eluted in fractions 91–96 and a smaller amount was found in fractions 65–78. Thus, the high molecular weight corrinoid–protein showed exactly the same elution pattern on the Sephadex G-100 as the low molecular corrinoid–protein of step VIII. Furthermore, the spectrum of fractions 91–96 was identical with that of the corrinoid–protein of step VIII. The molecular weight of the material eluted in fractions 65–78 is about 55,000 and is twice the molecular weight of that of fractions 91–96. It is our conclusion that the high molecular weight corrinoid–protein of step VI is a dimer of the corrinoid–protein of step VIII.

Results

Purity of the Corrinoid–Protein. The corrinoid–protein from step VIII was judged pure by several criteria. A single protein band was obtained during electrophoresis of the corrinoid–protein in polyacrylamide gel at pH 7.0, 8.3, and 8.9 (Figure 1). A slight trailing was observed behind the band at pH 8.9. Before staining a red band was visible on each gel, and it coincided with the protein band. Thus, the corrinoid moved with the protein during the electrophoresis at the three pH values. During gel chromatography on Sephadex G-100 or G-50 the ratio of protein and corrinoid was constant over the peak. This ratio was 26.9 mg of protein/μmol of corrinoid. Assuming one molecule of corrinoid per molecule of protein the molecular weight of the corrinoid–protein is 26,900. This value is in very good agreement with determinations of the molecular weight by other methods.

Molecular Weight of the Corrinoid–Protein. Several methods were employed to determine the molecular weight of the corrinoid–protein. Chromatography on Sephadex G-100 was performed with the following markers of known molecular weights: bovine serum albumin, 68,000; methylenetetrahydrofolate reductase, 55,000 (O'Brien *et al.*, 1973); ovalbumin, 43,000; and carbonic anhydrase, 27,000. Invariably the corrinoid–protein divided up into two components. In six different experiments the major portion, between 90 and 95% of the corrinoid–protein, eluted at a point corresponding to a mol wt of 28,000 ± 2,000. The second corrinoid–protein fraction eluted at a point corresponding to a mol wt of 57,000 ± 2,000. The corrinoid–protein of the higher molecular

TABLE II: Amino Acid Composition of the Corrinoid–Protein from *C. thermoaceticum*.

Amino Acid	No. of Residues[a]
Lysine	14
Histidine	2
Arginine	8
Aspartic acid	25
Threonine	11
Serine	12
Glutamic acid	33
Proline	9
Glycine	27
Alanine	27
Half-cystine	3
Valine	21
Methionine	11
Isoleucine	11
Leucine	25
Tyrosine	6
Phenylalanine	8
Tryptophan	0

[a] The numbers of residues are based on a protein with two histidine residues. The total number of residues is 254, giving a mol wt of 27,096.

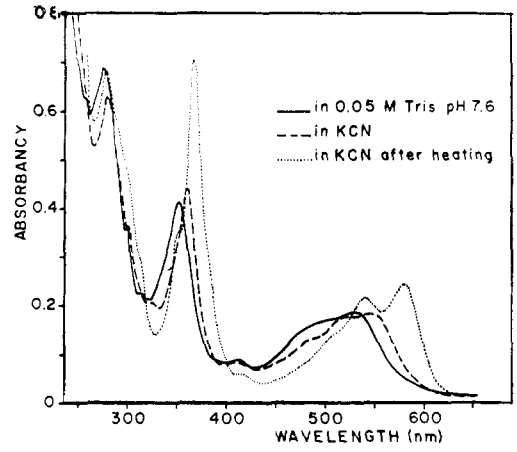

FIGURE 2: Absorption spectra of the corrinoid–protein from *C. thermoaceticum*. The solid line represents the spectrum of a solution of 0.6 mg/ml of corrinoid–protein in 0.05 M Tris, pH 7.6, in a light path of 1 cm. The dashed line shows the spectrum of the same solution after addition of solid potassium cyanide to obtain a pH between 10 and 11. The dotted line is the spectrum of the same solution after heating to 90° for 5 min.

weight apparently is a dimer of the smaller protein. The spectra of the two corrinoid–proteins are identical and during rechromatography of either of the corrinoid–proteins the two proteins were observed always with a ratio of monomer to dimer of about 10. Apparently an equilibrium between the dimer and the monomer exists, which favors the monomeric form.

The molecular weight of the corrinoid–protein was determined by dodecyl sulfate–polyacrylamide gel electrophoresis according to Weber and Osborn (1969). The corrinoid–protein moved as a single band with a mol wt of 26,800. This indicates that the corrinoid–protein consists of a single polypeptide chain.

Short column sedimentation equilibrium centrifugation of the corrinoid–protein was performed with a solution containing 1.2 mg/ml in 0.1 M Tris at pH 7.6. The rotor speed was 10,000 and the temperature 20° during the run, which was for 23 hr. The corrinoid–protein concentration was determined by scanning with the monochromator–scanner at 280 and 550 nm. At the latter wavelength the corrinoid moiety has an absorption. Plots of the logarithm of the protein concentration were a linear function of the square of the distance from the center of rotation at both wavelengths. Furthermore, the slopes of the lines in the two plots were almost identical, 0.147 and 0.146. These results show that the corrinoid–protein is homogeneous and that the corrinoid and the protein have identical behavior during centrifugation. The molecular weight obtained at 280 nm was 26,000 and at 550 nm was 26,200. We conclude from these results that the molecular weight of the corrinoid–protein is 27,000 ± 2,000.

Identification of the Corrinoid Moiety as 5-Methoxybenzimidazolylcobamide (Factor IIIm). To obtain the corrinoid moiety of the corrinoid–protein, about 15 mg of the protein was heated on a boiling water bath for 5 min in 2 ml of water containing 2 mg of potassium cyanide. The solution was applied to a Sephadex G-25 column (1.25 × 20 cm) which was eluted with water. The cyanocorrinoid was obtained free from the protein. The absorption spectra of the monocyano and the dicyano derivatives were identical with those of 5-methoxybenzimidazolylcobamide (Irion and Ljungdahl, 1965). Paper chromatography of the cyano derivative in solvents D, E, and F of Irion and Ljungdahl (1965) showed in all solvents a single corrinoid which chromatographed with 5-methoxybenzimidazolylcobamide. In a second experiment 10 mg of the corrinoid–protein was boiled in 2 ml of 80% ethanol for 10 min. The solution was applied to the Sephadex G-25 column. Some of the corrinoid remained with the protein but the main portion moved as a free corrinoid. The spectrum of this corrinoid was identical with (5-methoxybenzimidazolyl)hydroxy(aqua)cobamide (Irion and Ljungdahl, 1965). From the results of these experiments we have concluded that the corrinoid moiety of the corrinoid–protein is 5-methoxybenzimidazolylcobamide (factor IIIm) and that it is the only corrinoid bound to the protein.

Amino Acid Composition of the Corrinoid–Protein. Table II shows the amino acid composition of the corrinoid–protein. We were unable to find tryptophan. Histidine was the amino acid present in the smallest quantity and by assuming two molecules of histidine per molecule the protein was estimated to contain 254 amino acid residues and to have a mol wt of 27,096. Since the mol wt of 5-methoxybenzimidazolylcobamide is 1335, the total mol wt of the corrinoid–protein would be 28,431. This value is in good agreement with results obtained by gel chromatography, electrophoresis, and centrifugation experiments as well as by the direct determination of the amount of protein and corrinoid content. The partial specific volume calculated from the amino acid composition is 0.737 ml/g.

Absorption Spectra of the Corrinoid–Protein and the Reaction with Potassium Cyanide. The solid line in Figure 2 shows the absorption spectrum of the corrinoid–protein in 0.05 M Tris at pH 7.6. The dashed line is the spectrum obtained at pH 10, after addition of solid potassium cyanide to the cuvet. The dotted line shows the spectrum after the cuvet

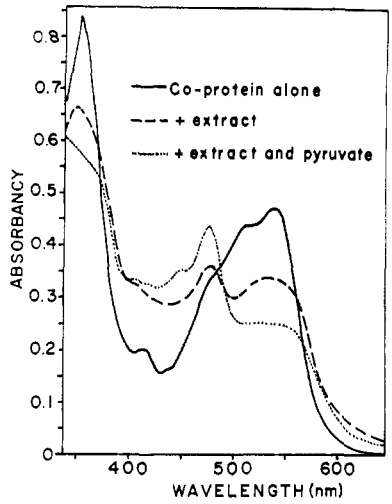

FIGURE 3: Reduction of the corrinoid–protein (Co-protein) by cell-free extract from *C. thermoaceticum*. The corrinoid–protein (0.06 μmol) of step VIII in 1 ml of 0.1 M Tris, pH 7.6, was placed in a cuvet, which was sealed with a serum stopper. The solution was made anaerobic by flushing with nitrogen, which was freed of oxygen by passage over a heated copper wire. An extract of *C. thermoaceticum* (0.05 ml; 1.5 mg of protein) was injected into the cuvet and after 10 min 0.1 ml of water containing 36 μmol of sodium pyruvate was added. The solid line shows the spectrum before addition of the extract. The dashed line is the spectrum 10 min after the addition of the extract and the dotted line is the spectrum 90 min after the addition of pyruvate. The incubation temperature was 25°.

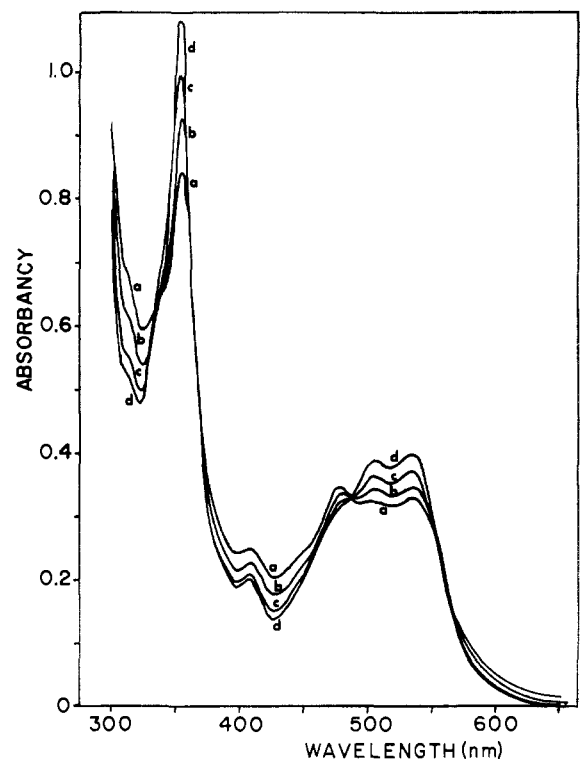

FIGURE 4: Change with time of the spectrum of the corrinoid–protein after precipitation with ammonium sulfate. Spectrum a is the corrinoid–protein in Tris, pH 7.6, from step VII before precipitation with ammonium sulfate. Spectra b, c, and d are from the same protein after being precipitated with ammonium sulfate and redissolved in the original volume of Tris, pH 7.6. Spectrum b was taken 20 min, c, 40 min, and d, 60 min after spectrum a.

containing the cyanide had been heated for 5 min at 90°. During the heating the corrinoid was released from the protein and converted to the dicyano form. The dotted line thus represents the spectrum of the free dicyanocorrinoid, with the exception of the high absorption at 275 nm of the protein still present in the cuvet. From this spectrum the amount of dicyano-5-methoxybenzimidazolylcobamide was determined using the absorption peaks at 580 nm, ϵ 10.13 $\times$ 10^3 M^{-1} cm^{-1}, and at 367 nm, ϵ 30.82 $\times$ 10^3 M^{-1} cm^{-1} (Friedrich, 1964). The values obtained were, at 580 nm, 0.0227 μmol of 5-methoxybenzimidazolylcobamide/ml and, at 367 nm, 0.0222 μmol. By using the average value of 0.0225 μmol of corrinoid/ml and a protein concentration of 0.6 mg/ml one obtains 26.7 mg of protein/μmol of the corrinoid. Assuming a mol wt of 27,000 and 1 μmol of 5-methoxybenzimidazolylcobamide/μmol of the corrinoid–protein, the molecular extinction coefficients for the corrinoid–protein were calculated from the solid curve of Figure 2. At the four absorption maxima the following values were obtained: ϵ_{530} 7.9 $\times$ 10^3; ϵ_{412} 3.6 $\times$ 10^3; ϵ_{351} 18.25 $\times$ 10^3; ϵ_{275} 30.5 $\times$ 10^3 M^{-1} cm^{-1}. In addition to the four absorption maxima the corrinoid–protein has shoulders at 500, 318, 300, and 260 nm. The spectrum in the region above 300 nm is almost identical with that of (5-methoxybenzimidazolyl)hydroxy(aqua)cobamide (Irion and Ljungdahl, 1965). The only difference is the height of the maximum at 351 nm, which is lower in the corrinoid–protein compared with the free corrinoid.

The corrinoid–protein reacts with cyanide at pH 10, as indicated by the change of the spectrum (dashed line in Figure 2). The shift of the spectrum above 300 nm indicates the formation of the monocyano derivative of 5-methoxybenzimidazolylcobamide (Irion and Ljungdahl, 1965). The peak at 361 nm, which corresponds to the peak at 351 nm of the hydroxy form, is again lower than that obtained with the free corrinoid.

At pH 10 in the presence of cyanide the free corrinoid forms the dicyano derivative. This is not formed with the corrinoid–protein unless the protein is denatured by heating. Apparently the protein is shielding the corrinoid moiety and prevents the reaction of the lower (5th) ligand with cyanide. In the reaction with cyanide at pH 10 the corrinoid is not released from the protein. Thus, the monocyanocorrinoid is still bound to the protein after gel filtration through Sephadex G-25.

Reduction of the Corrinoid–Protein. The spectrum represented by the solid line in Figure 3 appears to be that of the oxidized form of the corrinoid–protein. When the corrinoid is incubated under anaerobic conditions with an extract of *C. thermoaceticum* in the presence of pyruvate as a source of electrons, the spectrum shifts to that corresponding to a reduced corrinoid (B_{12r}) (Beaven and Johnson, 1955). This is shown in Figure 3 and is evident by the formation of a new absorption maximum at 475 nm and by the decrease of the maxima at 530 and 351 nm. The reduction of the corrinoid does not result in the release of the corrinoid from the protein. Thus the reduced corrinoid was eluted with the protein when applied to a Sephadex G-25 column. After passage through the column the spectrum of the corrinoid–protein was still that of the reduced form, although the chromatography was done without exclusion of air. Even after several days of storage under air the corrinoid–protein remained reduced and the spectrum was essentially that of the

dotted line in Figure 3. The reoxidation of the protein is apparently a very slow process.

Since *C. thermoaceticum* is grown under strict anaerobic conditions, it is likely that the corrinoid–protein exists within the cell at least partly in the reduced form. Furthermore, the observation that the reduced protein is oxidized only slowly should make it possible to isolate the reduced form of the protein without taking rigorous precautions to exclude air during the purification. Figure 4 shows evidence that the corrinoid–protein purified through step VII is at least partly reduced. Spectrum a is of the protein before precipitation with ammonium sulfate. There is an absorption maximum at 475 nm and the maxima at 530 and 351 nm are rather low compared with the maxima of the corrinoid–protein from step VIII (Figure 2). These are all indications that the corrinoid–protein of step VII is partly reduced. Electron paramagnetic resonance (epr) studies at −185° indicate also the presence of a Co^{2+}-bound corrinoid. A major resonance was obtained with $g_{\perp} = 2.037$. The line shape was similar but not identical with that of B_{12r} (cob(II)alamin) (Hogenkamp *et al.*, 1963).

Figure 4, curves b, c, and d, shows that precipitation with ammonium sulfate in some way renders the corrinoid–protein more susceptible to oxidation. Thus, the redissolved protein rather rapidly undergoes oxidation, which is evident by the disappearance of the peak at 475 nm and the increase of the maxima at 530 and 351 nm. About 1 day after the ammonium sulfate precipitation the spectrum of the corrinoid–protein is that of the solid line in Figure 2. Samples of the corrinoid–protein, which have not been precipitated with ammonium sulfate, have been stored for several months with free access to air. The spectra of these samples are still those of curve a in Figure 4.

Acidification of the Corrinoid–Protein. On acidification the 5-methoxybenzimidazolyl residue of free 5-methoxybenzimidazolylcobamide is protonated ($pK_a = 3.3$, Ljungdahl *et al.*, 1966) with the result that the bond between the base and the cobalt atom breaks. This can be observed spectrometrically; the maximum at 525 nm shifts to 460 nm and the spectrum at acid pH resembles that of corrinoids lacking the benzimidazole base. Spectra, of the corrinoid–protein, not shown, were recorded in 0.06 M acetic acid, pH 3.2, 0.04 M HCl, pH 1.55, and 0.1 M HCl, pH 1.0. The spectra at pH 3.2 and 1.55 were identical with the solid line in Figure 2. The spectrum in 0.1 M HCl was only slightly altered in that the peak at 351 nm was increased about 15% and the maximum at 534 nm was shifted slightly to 525 nm. Thus, acidification of the corrinoid–protein did not result in the dramatic shift of the maximum of 525 to 460 nm, which is observed with the free corrinoid. These results may be interpreted to mean that the protein is shielding the corrinoid, and prevents the protonation of the 5-methoxybenzimidazole base. Even at the lowest pH there was no sign of denaturation or precipitation of the corrinoid–protein.

Discussion

Methylcorrinoids have been shown to function in three systems: the cobamide-dependent formation of methionine, methane synthesis, and the synthesis of acetate from CO_2. The role of corrinoids in these reactions has recently been reviewed by Barker (1972). The formation of methionine from homocysteine and 5-methyltetrahydrofolate is catalyzed by a methyltransferase of mol wt 140,000. It contains 0.35–0.5 mol of tightly bound cobalamin/mol of protein (Stavrianopoulos and Jaenicke, 1967; Taylor and Weissbach, 1967). A corrinoid–protein of a mol wt between 100,000 and 200,000 is apparently involved in the formation of methane by *Methanosarcina barkeri* (Blaylock, 1968). Wood and Wolfe (1966) studying methane biosynthesis in *Methanobacillus omelianskii* have also isolated a corrinoid–protein which stimulated methane formation from 5-methyltetrahydrofolate. However, the role of corrinoids in the formation of methane with this organism is ambiguous since it has been shown to be a mixed culture of two organisms (Bryant *et al.*, 1967) and attempts have failed to demonstrate the corrinoid–protein in the methane-forming organism (McBride and Wolfe, 1971). The mol wt of the corrinoid–protein isolated from *C. thermoaceticum* is about 27,000. It contains 1 mol of 5-methoxybenzimidazolylcobamide/mol of protein. The protein also exists as a dimer with a mol wt of about 55,000, containing 2 mol of the corrinoid.

There is no direct evidence for a role of the corrinoid–protein described in this paper in the formation of acetate from CO_2 by *C. thermoaceticum*. However, it is tempting to postulate that it is involved in this synthesis. This idea is supported by circumstantial evidence. Thus, Poston *et al.* (1966) fractionated extracts from *C. thermoaceticum* by chromatography on DEAE-cellulose and demonstrated that a protein fraction, which was strongly adsorbed by DEAE-cellulose, was required for acetate synthesis from methylcobalamin. Ljungdahl *et al.* (1967) found that this fraction became labeled, when cell-free extracts were incubated with $^{14}CO_2$, and they showed that this fraction contained 5-methoxybenzimidazolylcobamide and a radioactive derivative of this corrinoid.

C. thermoaceticum contains more than 20 different corrinoids with cobyric acid as the most abundant (Ljungdahl *et al.*, 1966). Several of these have been isolated as their *Co*-methyl derivatives (Irion and Ljungdahl, 1968), which may indicate that they may participate as methyl carriers in the synthesis of acetate from CO_2. It is therefore somewhat surprising to find that the corrinoid–protein contains only 5-methoxybenzimidazolylcobamide. This is the main corrinoid containing a base in *C. thermoaceticum*. Apparently the protein selectively binds 5-methoxybenzimidazolylcobamide and perhaps the base is important for the binding of the corrinoid to the protein. One kilogram of frozen cells of *C. thermoaceticum* contains between 300 and 700 μmol of corrinoids, of which 13.5% is 5-methoxybenzimidazolylcobamide (Ljungdahl *et al.*, 1966). Assuming that the 17.6 μmol of corrinoid, which was found in step IV, Table I, constitutes the corrinoid–protein, it contains between 3 and 7% of the total amount of corrinoids of *C. thermoaceticum* and almost half of the amount of 5-methoxybenzimidazolylcobamide.

The binding of the corrinoid to the protein imposes restrictions on the reactivity of the corrinoid. Thus, at pH 10 it reacts only with 1 mol of cyanide while under the same conditions the dicyano derivative is formed with the free corrinoid. At acid pH the base of the free corrinoid is protonated and the coordinate linkage between the base and the cobalt atom is broken. This reaction has not been observed with the corrinoid–protein even at pH 1. As judged from spectrophotometric and epr studies it is likely that the corrinoid in the corrinoid–protein exists in a reduced form corresponding to B_{12r} (cob(II)alamin), and that it is quite stable toward oxidation by air. However, precipitation with ammonium sulfate seems to induce a modification of the corrinoid–protein. After the precipitation the corrinoid is susceptible to oxidation to the hydroxy(aqua) derivative. Taylor and Weissbach (1967) and Taylor (1970) also found that the corrinoid

in 5-methyltetrahydrofolate–homocysteine transferase may exist in the B_{12r} form, which is stable to oxygen.

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