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Nickel Tetrapyrrole Cofactor F_{430}: Comparison of the Forms Bound to Methyl Coenzyme M Reductase and Protein Free in Cells of *Methanobacterium thermoautotrophicum* ΔH†

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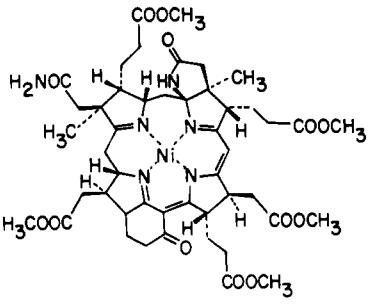
ABSTRACT: The nickel tetrapyrrole cofactor F_{430} occurs in two intracellular forms in *Methanobacterium thermoautotrophicum* ΔH. One form is bound to the methyl coenzyme M reductase as previously described [Ellefson, W. L., Whitman, W. B., & Wolfe, R. S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3707–3710]. A simple high-yield purification of this enzyme is described. A second form, protein-free F_{430}, was purified by using ion-exchange, gel filtration, and reverse-phase chromatography. The protein-bound F_{430} was released from the pure enzyme by using gentle extraction procedures, and the two forms were compared. The extracted and protein-free species were identical by ultraviolet–visible spectroscopy, reverse-phase high-performance liquid chromatography elution position, coenzyme M analysis, and mass spectrometry. Our data suggest that the complete F_{430} molecule is the free pentaacid form (m/z = 905) of the F_{430} structure proposed by Pfaltz et al. [Pfaltz, A., Jaun, B., Fassler, A., Eschenmoser, A., Jaenchen, R., Gilles, H. H., Diekert, G., & Thauer, R. K. (1982) *Helv. Chim. Acta 65*, 828–865]. F_{430} does not possess covalently bound coenzyme M or lumazine derivatives.

In 1978, Gunsalus & Wolfe (1978) first reported the isolation of a yellow, nonfluorescent chromophore from heat-treated extracts of *Methanobacterium thermoautotrophicum* ΔH. This compound, named factor F_{430}[1] because of its absorbance maximum at 430 nm, has since been observed in all methanogens examined (Diekert et al., 1981). F_{430} was recently shown to be the chromophore in methyl-*S*-CoM reductase (Ellefson et al., 1982). This enzyme catalyzes the last step in methanogenesis, the two-electron reductive cleavage of methyl-*S*-CoM (methyl mercaptoethanesulfonate) to methane and HSCoM (coenzyme M, mercaptoethanesulfonate) (Ellefson & Wolfe, 1980; Nagle & Wolfe, 1983a). Enzyme-bound

$$CH_3S\wedge SO_3^- + 2H^+ + 2e^- \longrightarrow CH_4 + HS\wedge SO_3^-$$

factor F_{430} exhibited an absorbance peak at 422 nm and a 445-nm shoulder (Ellefson et al., 1982). Boiling methanol extraction of the enzyme released the chromophore as the 430-nm-absorbing species with a stoichiometry of 1.75 mol of F_{430} bound per 1 mol of protein (M_r 300 000) (Ellefson et al., 1982). The role of F_{430} in catalysis by the methyl-*S*-CoM reductase has not been elucidated.

F_{430} is the only known biological example of a nickel–tetrapyrrole complex (Diekert et al., 1980a,b; Pfaltz et al., 1982). The structure for the methanolysis product of F_{430} has recently been established by Pfaltz et al. (1982). As shown below (compound I), the chromophore is a highly reduced, tetrahydro derivative of the corphin system—a structural hybrid of porphyrins and corrins. The ligand skeleton is that



I

of a uroporphinoid (type III) with an additional carbocyclic ring. Although the macrocyclic ring structure is now fully established in $HClO_4$-extracted F_{430} (Pfaltz et al., 1982), it has been claimed by others that there are additional components associated with the native cofactor in cells. Vogels and co-workers have purified a series of incompletely defined F_{430} species differing in redox state or associated components. They suggested that native F_{430} includes bound HSCoM (Keltjens et al., 1982), a lumazine derivative (Keltjens et al., 1983a), and perhaps other substituents (Keltjens et al., 1983b). Furthermore, in collaboration with Wolfe and colleagues, they found HSCoM was associated in stoichiometric amounts with extracted F_{430} from pure methyl-*S*-CoM reductase (Keltjens et al., 1982).

In this paper, we demonstrate that for *M. thermoautotrophicum* ΔH grown on 1 μM Ni, F_{430} exists in two pools. These pools include the enzyme-bound species discussed by Ellefson et al. (1982) and a previously unreported protein-free form.

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[1] Abbreviations: F_{430}, nickel tetrapyrrole cofactor of methanogenic bacteria; HSCoM, 2-mercaptoethanesulfonic acid (coenzyme M); DE52, diethylaminoethylcellulose; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; KP_i, potassium phosphate.

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The two species of F_{430} were purified, compared, and found each to be consistent with the F_{430} pentaacid. Proof of this structure has now been independently established by the Eschenmoser and Thauer groups (D. A. Livingston, A. Pfaltz, J. Schreiber, A. Eschenmoser, D. Ankel-Fuchs, J. Moll, R. Jaenchen, and R. K. Thauer, unpublished results).

Experimental Procedures

Materials. DEAE-cellulose (DE52), Sephadex G-50, Sephadex G-25, and phenyl-Sepharose were purchased from Pharmacia. HPLC-grade methanol and water were obtained from MCB Reagents.

Preparation of Crude Extract. M. thermoautotrophicum ΔH was cultivated at 62 °C in media containing 1 μM Ni as described by Schonheit et al. (1979) using a New Brunswick 25-L fermenter. Cells grown under an atmosphere of 80% H_2/20% CO_2 to an OD_{660} of over 1 were bubbled for 45 min with 100% CO_2 and then concentrated by using a Millipore Pellicon membrane filtration apparatus. Harvested cells were stored as a frozen paste at −70 °C. A suspension of cells in pH 7.0 50 mM KP_i buffer (1 g of cells/1.5 mL of buffer) was passed through a French pressure cell at 19 000 psi and centrifuged at 30000*g* for 30 min at 4 °C to yield the crude extract.

Preparation of Methyl-S-CoM Reductase. All enzyme purification steps were performed at 4 °C. Crude extract (69 mL, 37 mg/mL) was chromatographed on a column (2.5 × 10 cm) of DE52 equilibrated in 50 mM KP_i, pH 7.0, buffer. The resin was eluted sequentially with buffer containing 0.0, 0.15, and 0.35 M KCl. The 0.15 M salt step eluted protein-free F_{430} which is discussed in the following section. Buffer containing 0.35 M KCl eluted a brown band comprised of several proteins including the methyl-*S*-CoM reductase. The concentration of KCl was adjusted to 1 M KCl, and the fraction was chromatographed on a column (2.5 × 14 cm) of phenyl-Sepharose equilibrated in this buffer. Methyl-*S*-CoM reductase exhibited no interaction with the phenyl-Sepharose and was collected in the void volume. After concentration by Amicon pressure filtration using a PM30 membrane, the enzyme pool was desalted on a column (2.5 × 15 cm) of Sephadex G-50 to yield homogeneous methyl-*S*-CoM reductase as determined by SDS–polyacrylamide gel electrophoresis.

Preparation of the Protein-Free Form of F_{430}. The yellow pool eluting from DE52 resin with 0.15 M KCl buffer was further fractionated at 4 °C by using a column (2.5 × 50 cm) of Sephadex G-25 equilibrated in 50 mM ammonium acetate. This gel filtration step was used to resolve the pool into protein, F_{430}, and small molecular weight fractions. F_{430} was then isolated by reverse-phase chromatography using a 10-μm C-18 column (Waters Associates, 0.46 × 25 cm, equilibrated in 50 mM ammonium formate, pH 7.0, 1.5 mL/min). A 25-min linear gradient from 0.0% to 20% CH_3OH was used to elute F_{430}. An additional 10-min linear gradient from 20% to 50% CH_3OH eluted contaminating cofactors. A Du Pont Instruments HPLC equipped with a series 8800 gradient controller, an 850 absorbance detector, and a Micromeritics 786 variable-wavelength detector was used throughout this study.

Extraction of Enzyme-Bound F_{430}. F_{430} was extracted from crude cell preparations and from DE52 fractions by using the $HClO_4$ method of Diekert et al. (1981). This extract was neutralized, and the F_{430} was quantitated by integration of the HPLC absorbance peak (436 nm) using a Hewlett Packard 3390A integrator.

Homogeneous methyl-*S*-CoM reductase was denatured to dissociate the cofactor F_{430} by using three methods: $HClO_4$ extraction (Diekert et al., 1981), ethanol extraction, or freeze–thaw dissociation.[2] For solvent extraction, the protein solution was carefully adjusted to 0.6 M KCl and 40% ethanol, resulting in slow release of cofactor. High salt concentration was required for effective extraction. After several days at 4 °C, protein was removed by centrifugation and Amicon filtration to yield free cofactor solution. Alternatively, enzyme frozen in buffer containing 1 M NaCl partially precipitated and released F_{430} upon thawing. Centrifugation and gel filtration removed the cofactor from the apoprotein.

Analytical Methods. UV–visible spectroscopy was performed by using a Perkin-Elmer Lambda 3 or Lambda 5 spectrophotometer. Ni was quantitated with a Perkin-Elmer 2380 atomic absorption spectrophotometer fitted with an HGA-400 graphite furnace assembly. SDS–polyacrylamide gel electrophoresis (10% gel) was performed by using the method of Laemmli (1970). Coenzyme M determinations were performed by Dr. David Nagle and Dr. Jack Jones using the bioassay developed by Balch & Wolfe (1976). Fast atom bombardment mass spectrometry was performed by using a Finnigan MAT 731 mass spectrometer equipped with an Ion Tech atom gun.

Results

Separation of Protein-Free and Protein-Bound F_{430}. DE52 ion-exchange chromatography of crude extract from *M. thermoautotrophicum* ΔH resolved two F_{430} fractions. One pool was further purified as F_{430} bound to methyl-*S*-CoM reductase, previously described by Ellefson et al. (1982). The second pool possessed the protein-free F_{430}. These two forms of F_{430} were also readily separated by gel filtration chromatography of the crude extract using Sephadex G-50.

The relative amounts of F_{430} in the two fractions were compared by $HClO_4$ extraction and HPLC analysis. Of the total F_{430} in the cells, from 10 to 50% was bound to the methyl-*S*-CoM reductase (five preparations, five different cell growths).

Purification and Characterization of Methyl-S-CoM Reductase. A simple, three-step purification procedure was used to obtain homogeneous methyl-*S*-CoM reductase. The protocol used DE52 and phenyl-Sepharose chromatography, similar to the method recently noted by Nagle & Wolfe (1983b), followed by Sephadex G-50 chromatography. The hydrophobic resin bound all contaminating proteins in the DE52 fraction, but not the reductase itself. Contaminants of small molecular weight were removed by gel filtration chromatography. The amount of enzyme prepared (150 mg from 20 g of wet cells) corresponds to 7% of the protein in the crude extract. This compares to a value of 12% of the cellular protein estimated by Ellefson & Wolfe (1981).

The methyl-*S*-CoM reductase was homogeneous by SDS–polyacrylamide gel electrophoresis. Three subunits of M_r 68 000, 47 000, and 38 000 were observed, in close agreement with those (M_r 68 000, 45 000, and 38 500) reported by Ellefson & Wolfe (1981) but in contrast to the findings (M_r 61 000, 50 000, and 36 000) of Moura et al. (1983). We observed that in some preparations a substoichiometric contaminant appeared at M_r 30 000. Hartzell & Wolfe (1983) recently reported that the four-subunit methyl-*S*-CoM reductase from *M. barkeri* possesses a subunit of nearly this size, M_r 28 000.

The UV–visible spectrum of our purified methyl-*S*-CoM reductase (Figure 1) was similar to that reported (Ellefson et al., 1982; Moura et al., 1983); however, there was a distinct

[2] Hydrolysis of an especially labile bond cannot be excluded by these procedures. Professor R. Thauer (unpublished results) has developed a similar solvent extraction method using 80% ethanol and 2 N LiCl.

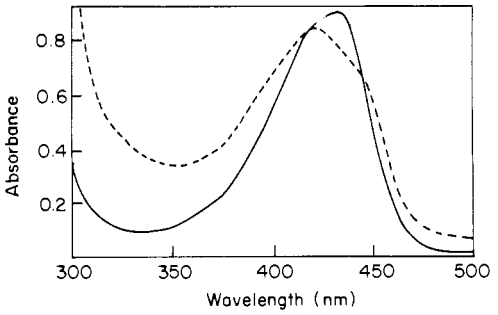


FIGURE 1: UV–visible absorbance spectra of enzyme-bound and protein-free F_{430}. Spectrum of methyl-*S*-CoM reductase, 5.9 mg/mL (37.8 μM coenzyme) in 50 mM ammonium formate buffer, pH 7.0 (---); spectrum of protein-free F_{430}, 39.1 μM in 50 mM ammonium formate buffer, pH 7.0 (—).

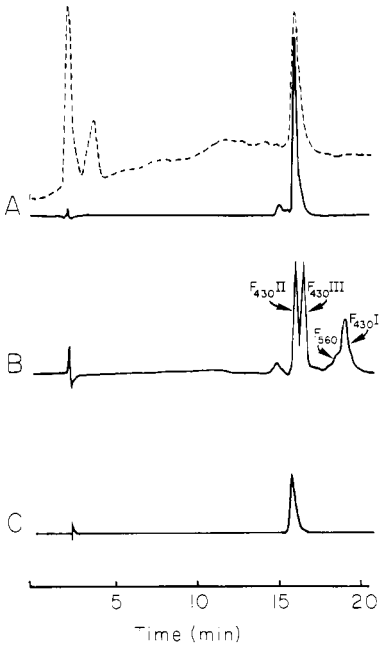


FIGURE 2: Reverse-phase HPLC absorbance profiles of F_{430} samples. Sample preparation and HPLC conditions are described in the text. (A) Protein-free F_{430} pool (0.2 mL) after Sephadex G-25 chromatography; absorbance at 214 nm (---) and at 436 nm (—). (B) Same sample (0.1 mL) after heating 60 min in a boiling water bath; absorbance at 436 nm. (C) Ethanol-extracted F_{430} (0.05 mL); absorbance at 436 nm.

blue shift to yield a peak at 418 nm and a shoulder at 445 nm. Samples of the methyl-*S*-CoM reductase possessed 1.4–1.9 mol of Ni per mol of enzyme (M_r 300 000). An extinction coefficient of 23 100 M^{-1} cm^{-1} at 418 nm was calculated for the enzyme-bound F_{430} on the basis of Ni analysis. Bioassay (Balch & Wolfe, 1976) revealed the presence of stoichiometric amounts of coenzyme M in the pure methyl-*S*-CoM reductase (1.06 ± 0.15 HSCoM/F_{430}, N = 8).

Purification of Protein-Free F_{430}. The yellow band recovered from DE52 after elution with 0.15 M KCl buffer was further chromatographed on Sephadex G-25 to resolve F_{430} from protein and small molecular weight contaminants. Final purification of protein-free F_{430} was achieved by using HPLC as shown in Figure 2A. This HPLC system was capable of resolving F_{430} from other cofactors found in methanogens (e.g., flavins, deazaflavins, methanopterins, etc.). In addition, thermally damaged forms of F_{430} were separated as seen in Figure 2B. Four species of F_{430} generated by heat treatment were isolated and found to possess spectroscopic characteristics similar to F_{430} I, F_{430} II, F_{430} III, and F_{560} reported by Diekert et al. (1980a). To preclude thermal damage of protein-free F_{430}, all purification steps except HPLC were carried out at 4 °C.

Table I: Fast Atom Bombardment Mass Spectrometry of F_{430}

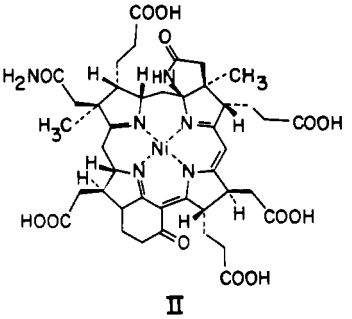
	m/z							
	903	904	905	906	908	908	909	910
protein-free F_{430}	33	36	100	77	74	52	36	24
extracted F_{430}[a]	22	30	100	75	63	50	29	20

[a] These data were obtained for solvent-extracted F_{430}. Comparable results were obtained for $HClO_4$ or high salt, freeze-thaw-extracted samples.

The isolated protein-free F_{430} had a UV–visible spectrum (Figure 1) identical with that reported previously (Gunsalus & Wolfe, 1978; Pfaltz et al., 1982), with peaks at 275 and 430 nm and a slight shoulder at 295 nm. The calculated extinction coefficient of 23 300 M^{-1} cm^{-1} based on Ni analysis was in close agreement with reported values (Diekert et al., 1980a,b). The DE52 pool eluted with 0.15 M KCl had 0.32 HSCoM per Ni; however, subsequent purification steps removed all HSCoM as determined by bioassay. Fast atom bombardment mass spectroscopy showed a single nickel-containing species with m/z = 905 (see Table I).

Extraction of Protein-Bound F_{430}. F_{430} was released from methyl-*S*-CoM reductase by using $HClO_4$, freeze–thaw treatment, or a solvent extraction procedure to minimize decomposition. The chromophore released by these methods was compared to the protein-free F_{430}. The extracted samples coeluted with protein-free F_{430} on reverse-phase HPLC (see Figure 2C). Bioassay of HPLC-purified samples indicated an absence of coenzyme M (≤0.012 HSCoM/F_{430}). In addition, UV–visible spectroscopy was unable to distinguish between the various F_{430} samples.

Altered forms of F_{430} may show no spectroscopic changes and may coelute on HPLC. Thus, mass spectrometry was used as a third method to test for differences in the F_{430} samples. Mass spectrometry of the methanolysis product from $HClO_4$ extraction yielded an m/z = 975 cation (Pfaltz et al., 1982). A nonesterified sample is calculated to have m/z = 905. The F_{430} extracted from enzyme by using the gentle, freeze–thaw, or ethanol extraction procedures had the same value, i.e., m/z = 905 (see Table I). Both extraction methods resulted in an F_{430} cation identical with that seen in the protein-free form. The mass of these species is consistent with the mass calculated for compound II, the pentaacid derivative of the corphin proposed by Pfaltz et al. (1982).



II

Discussion

The macrocyclic ring structure for the methanolysis product of $HClO_4$-extracted F_{430} has been established by Thauer, Eschenmoser, and colleagues (Pfaltz et al., 1982). In addition, Vogels and co-workers suggested that HSCoM (Keltjens et al., 1982), tetrahydrolumazine (Keltjens et al., 1983a), and other substituents (Keltjens et al., 1983b) are bound in an

undetermined manner to the chromophoric ring system. To further define the F_{430} cofactor, we have purified F_{430} at physiological pH, at low temperature, and with mild extraction procedures in hopes of retaining the native structure. We observed that two pools of F_{430} were present in the cell, viz., F_{430} bound to methyl-*S*-CoM reductase (Ellefson et al., 1982) and a previously undocumented protein-free form.

Methyl-*S*-CoM reductase accounts for up to 12% of the cellular protein (Ellefson & Wolfe, 1981) and is thought to have 2 mol of F_{430} bound per mol of enzyme (Ellefson et al., 1982). The coenzyme is very tightly bound to the enzyme as demonstrated by the retention of activity after dialysis or gel filtration (Ellefson & Wolfe, 1981). Our enzyme preparation was somewhat deficient in cofactor (1.4–1.9 F_{430}'s per enzyme molecule); however, the levels of free F_{430} exceeded the depleted chromophore level by severalfold. Thus, the protein-free F_{430} is *not* merely a result of cofactor dissociation from methyl-*S*-CoM reductase. High Ni levels in the growth media may lead to an overproduction of the corphinoid (Diekert et al., 1980a) in the protein-free state. Protein-free F_{430} could then be incorporated into methyl-*S*-CoM reductase.

It was possible that incorporation of F_{430} into protein may involve a chemical change in the cofactor structure. Precedents for such a change include modifications of riboflavin, vitamin B_{12} (most notably), and pantothenic acid to form FAD, coenzyme B_{12}, and coenzyme A, respectively. To test for chemical differences in the two species of F_{430}, the chromophore was extracted from methyl-*S*-CoM reductase. The literature extraction procedures include heat treatment (Gunsalus & Wolfe, 1978; Keltjens et al., 1982, 1983a,b) or $HClO_4$ denaturation at pH 2 (Diekert et al., 1980b, 1981; Pfaltz et al., 1982). Diekert et al. (1981) first demonstrated that factor F_{430} is thermally unstable (see also Figure 2B), precluding use of high temperatures for extraction. Furthermore, $HClO_4$ treatment may hydrolyze a labile bond. We have developed gentle, freeze–thaw, and ethanol extraction procedures[2] to release the cofactor, thus circumventing the harsh extraction methods. The F_{430} released by these extraction methods is identical with the protein-free form of F_{430} on the basis of UV–visible spectroscopy, HPLC elution position, HSCoM bioassay, and the m/z of the cation from FAB mass spectrometry. Pfaltz et al. (1982) found the methylated derivative of F_{430} to have a cation with $m/z = 975$. Our data on extracted F_{430} are consistent with the pentaacid derivative (compound II) of this structure [$m/z = 975 - 5(14) = 905$] as the coenzyme form. There is no need to pose a stable covalent link of HSCoM or other components to F_{430}.

Identical conclusions have independently been reached on the structure of F_{430} in cells of *M. thermoautotrophicum*, Marburg strain, by the Eschenmoser and Thauer groups. In that work, F_{430} was eluted from reductase (which had been purified by ethanol fractionation) by using 2 N LiCl in 80% ethanol and purified by reverse-phase HPLC, and likewise shows an m/z of 905 by FAB mass spectrometry. In addition, they have conclusively established the structure of the pentaacid as compound II by CD, IR, and ^{13}C NMR spectroscopy (D. A. Livingston et al., unpublished results). It seems reasonable to term the pentaacid II then as coenyzme F_{430}.

Although no covalent links to other components are likely, coenzyme F_{430} may be physically associated with other components in the methyl-*S*-CoM reductase active site. Indeed, we and others (Keltjens et al., 1982; Nagle & Wolfe, 1983b) have observed stoichiometric amounts of HSCoM which copurified with the enzyme. The interrelationships of these bound components and the mechanism of catalysis for methyl-*S*-CoM reductase remain to be elucidated.

Acknowledgments

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Registry No. Cofactor F_{430}, 73145-13-8; methyl-*S*-CoM reductase, 53060-41-6.

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