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Biochemistry

Nickel-containing factor F_{430}: Chromophore of the methylreductase of *Methanobacterium*

(tetrapyrrole/methanogenesis)

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ABSTRACT **The yellow chromophore of the methyl-coenzyme M methylreductase of *Methanobacterium thermoautotrophicum* has been found to be the nickel-containing factor F_{430}. Treatment of ^{63}Ni-labeled methylreductase with 80% aqueous methanol released the radiolabel as well as the yellow chromophore; both properties were associated with a single compound that was found to be identical to F_{430}, the stoichiometry being 1 mol of nickel per mol of F_{430} and 2 mol of F_{430} per mol of methylreductase.**

The role of nickel in biological systems is not well understood. Nickel is required for production of an active urease in plants and bacteria (1, 2), for synthesis of an active hydrogenase in Knallgas bacteria (3, 4), and for synthesis of carbon monoxide dehydrogense in acetogenic bacteria and some clostridia (5, 6). Nickel also is required for growth of methanogenic bacteria (7, 8) and for synthesis of an abundant yellow compound (9) found in cell extracts, named factor 430 (F_{430}) because of an absorption maximum at 430 nm. F_{430} is nonfluorescent (10) and contains stoichiometric amounts of nickel (11, 12). Evidence showing the incorporation of δ-aminolevulinic acid and succinate into F_{430} suggests that it is a tetrapyrrole (13, 14), the first nickel-containing tetrapyrrole to be found in living organisms.

In spite of interest in F_{430}, there has been no information available concerning its function. The methylreductase from *Methanobacterium thermoautotrophicum* recently has been purified to homogeneity (15). The presence of a nonfluorescent yellow color in the purified methylreductase (15) led us to explore the possibility that this chromophore might be F_{430}; here, we identify this unique compound as the chromophore of the methylreductase.

MATERIALS AND METHODS

Chemicals and Materials. $^{63}NiCl_2$ in 1 M HCl (11.1 Ci/g; 1 Ci = 3.7×10^{10} becquerels) was purchased from ICN Radiochemicals. A nickel standard was obtained from Scientific Products. We used DEAE-cellulose (DE52) and silica gel plates (LK5) from Whatman, acrylamide and *N,N'*-methylenebisacrylamide from Bio-Rad, cellulose plates from Eastman Kodak, Sephadex ion exchange resins from Pharmacia, and agarose from Miles.

Culture of Cells and Enzyme Preparation. Growth of *M. thermoautotrophicum*, preparation of cell-free extracts, purification of the methylreductase, preparation of antibodies to the methylreductase, and purification of F_{430} were carried out as described (12, 15–17). The inoculum for experiments with labeled nickel was grown for two transfers in basal medium without exogenous nickel. A 5-ml portion was used to inoculate 150 ml of medium and then 7.5 μl of a solution containing 30.7 μCi of ^{63}Ni and 32.8 μg of $NiCl_2$ was added. Nickel solutions were filter sterilized. Cultures were grown in 80% H_2/20% CO_2 at 65°C using standard pressurized atmosphere techniques (17). When the rate of hydrogen consumption decreased, the cells were harvested aerobically, and the methylreductase was purified. Aliquots of medium taken before and after growth were used to calculate the initial specific radioactivity of the medium and the percentage of label uptake.

Preparation of the Methylreductase Chromophore. All operations were carried out aerobically in dim light. To a solution of homogeneous methylreductase, sufficient boiling methanol was added to yield an 80% aqueous methanol solution. Immediately after the addition of boiling methanol, the extract was placed in an ice bath for 0.5 hr, and then it was centrifuged at 15,000 × *g* for 15 min. The supernatant solution was saved. The pellet was suspended in 80% aqueous methanol and this suspension was centrifuged. The supernatants were combined, methanol was removed under a stream of nitrogen, and the residue was lyophilized. The lyophilized extract was suspended in water and centrifuged. The supernatant solution was saved for subsequent analysis. Atomic absorption spectroscopy and TLC on silcia gel plates were carried out as described (12). TLC on cellulose plates was carried out using 60% aqueous 2-propanol as solvent. To assay for radioactivity, each 0.5-cm section of a thin-layer plate was removed and transferred to a separate scintillation vial. To each vial, 0.2 ml of 0.25 M NaCl solution was added, and the vial was incubated overnight at room temperature to extract the chromophore prior to assay. HPLC was carried out as described (12) except for the substitution of an UV detector.

Additional Measurements and Methods. Prior to protein analysis (18), the enzyme was precipitated with cold 15% trichloroacetic acid and centrifuged; the pellet was washed three times with acetone to remove 2-mercaptoethanol and trichloroacetic acid. Nondenaturing polyacrylamide gel electrophoresis was carried out as described (15). Slab gels were stained for protein (15) or sliced into 2-mm sections and assayed for protein-bound radioactivity. Each section was soaked in 0.1 M NaCl at room temperature overnight prior to addition of scintillation cocktail. Immunodiffusion was carried out as described (15), except that the plate was photographed after 48 hr and the agar was dried without washing. The dried preparation was placed against Kodak XR-5 film for 4 days at −70°C. Scintillation assay was carried out on a Packard Tricarb 460 CD using 75% toluene/25% Triton X-100 (vol/vol)/0.6% diphenyloxazole as the scintillation cocktail.

RESULTS

Identification of F_{430} as the Chromophore of the Methylreductase. Purified methylreductase from *M. thermoautotro-*

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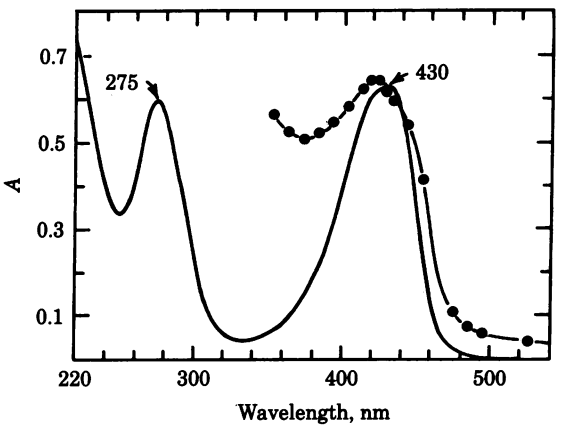

FIG. 1. Visible spectrum of purified methylreductase and HPLC-purified chromophore. Purified methylreductase was diluted with 0.05 M Tris (pH 7) to give a final concentration of 30 μM in 0.1 ml. The visible spectrum was recorded on a Cary 219 spectrophotometer with baseline correction (●). The methylreductase chromophore was extracted and purified by HPLC. HPLC-purified chromophore was concentrated by lyophilization and then suspended in 0.05 M Tris (pH 7.0). The concentration of chromophore, based on an extinction coefficient for purified F_{430} of 21,000 $M^{-1}\cdot cm^{-1}$ (12), was equal to 32 μM F_{430} (——).

phicum exhibited a characteristic nonfluorescent yellow color; the visible spectrum is shown in Fig. 1. The protein had an absorption maximum at 422 nm and a shoulder at 445 nm. Treatment of the protein with 80% boiling methanol released a yellow compound that, after further purification by HPLC, had a visible/UV spectrum identical to that of F_{430} (10). On the basis of a molar extinction coefficient of 21,000 $M^{-1}\cdot cm^{-1}$ for F_{430} (12) and a molecular weight of 300,000 for the methylreductase (15), 1.75 mol of F_{430} was released from 1.0 mol of the methylreductase by this method.

The chromatographic properties of the methanol-extracted chromophore, the purified chromophore, and F_{430} are compared in Table 1. All three compounds behaved similarly on cellulose sheets developed in 60% 2-propanol and on silica gel plates. The methanol-extracted chromophore and F_{430} eluted from the HPLC column at nearly identical retention times. These data suggest that the chromophore isolated from the methylreductase is F_{430}.

Identification of Protein-Bound Nickel in the Methylreductase. Since F_{430} contains nickel (12), it was of interest to determine whether or not the methylreductase was a nickel metalloprotein. To obtain evidence on this point, the homogeneous protein was analyzed by atomic absorption spectroscopy, and the methylreductase from cells grown in the presence of ^{63}Ni was isolated. The results of the atomic absorption study are given in Table 2. The mean nickel/protein ratio (mol/mol) was 1.92, and the range was 1.88–1.92. Growth of *M. thermoautotrophicum* on 31 μCi of $^{63}NiCl_2$ resulted in uptake of 25% of the added label. When the cells were broken and the methylreductase was purified, label was found to comigrate with the protein on nondenaturing gel electrophoresis (Fig. 2). Furthermore, the ^{63}Ni-labeled methylreductase was antigenically identical to the purified unlabeled methylreductase used for the chromophore isolation and atomic absorption studies (Fig. 3). Autoradiography of the immunodiffusion plate showed that the precipitation lines observed visually also contained radioactive nickel. Only limiting amounts of ^{63}Ni-labeled homogeneous enzyme were available, and the suboptimal concentration of protein used in the methylreductase assay produced a specific activity 20% of the normal level. Analysis of the specific radioactivity of the initial medium and the incorporation of label into the methylreductase gave the stoichiometry given in Table 2. The labeled methylreductase contained 2.0 mol of nickel per

Table 1. Chromatographic properties of methylreductase chromophore

Sample	R_F on TLC System 1	R_F on TLC System 2	HPLC retention time, min
Chromophore			
Methanol extracted	0.80	0.33	12.0
After HPLC	0.81	0.33	—
F_{430}	0.81	0.34	11.9

The yellow chromophore from 20 mg of purified methylreductase was extracted with methanol and purified by HPLC. The dried chromophore was suspended in 100 μl of H_2O and a 10-μl aliquot was used as the sample. A 2-μl sample of a 0.01 M solution of F_{430} was used as the standard. TLC was carried out at 25°C. In system 1, cellulose sheets were used and the solvent was 60% 2-propanol. In system 2, silica gel was used and the solvent was methyl acetate/butanone/formic acid/H_2O, 5:3:1:1 (vol/vol). HPLC was on a 0.39 × 30 cm C_{18} μBondapak column, and the solvent was 30% methanol/1% formic acid adjusted to pH 3.0 with NH_4OH.

Table 2. Stoichiometry of nickel in methylreductase

Method of analysis	Protein, nmol/ml	Ni, nmol/ml	Ratio
Atomic absorption	31.7	60.5	1.91
	31.7	62.1	1.96
	60.0	113.0	1.88
Mean			1.92
Purification of ^{63}Ni-labeled enzyme			
Preparation 1	0.88	1.78	2.02
Preparation 2	0.29	0.58	2.00
Preparation 3	0.63	1.21	1.92
Preparation 4	1.33	2.75	2.07
Mean			2.00

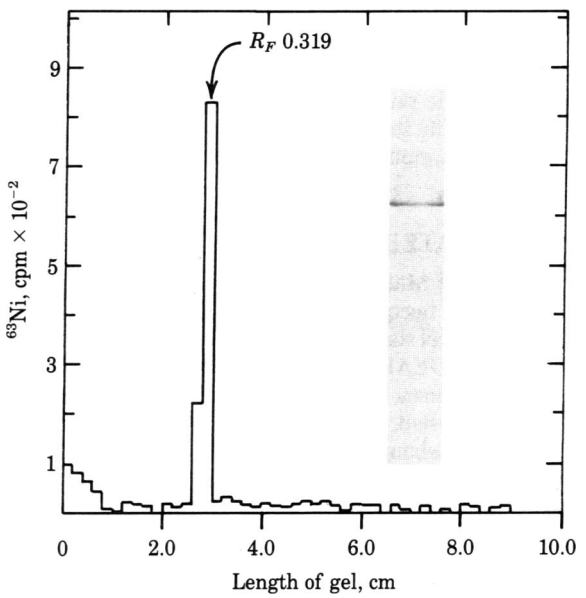

FIG. 2. Nondenaturing polyacrylamide gel electrophoresis of purified ^{63}Ni-labeled methylreductase. Samples containing 6.2 μg of purified ^{63}Ni-labeled methylreductase were subjected to electrophoresis in adjacent lanes. Upon completion, the slab gel was removed and the gel was cut along the appropriate lanes. One lane was stained for protein (*Inset*), and the other was sectioned for radioactivity assay. Approximately 52% of added radioactivity was recovered.

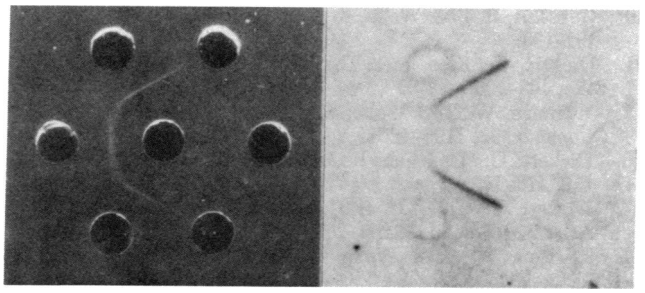

FIG. 3. Immunodiffusion of ^{63}Ni-labeled and unlabeled methylreductase. The immunoprecipitation pattern is shown on the left and an autoradiogram of the dried gel is shown on the right. The wells (clockwise from the upper right) contained 5 μg of bovine serum albumin and 3 μg of rubredoxin (*M. thermoautotrophicum*), 5 μg of bovine serum albumin, 5 μg of ^{63}Ni-labeled methylreductase, 6.6 μg of unlabeled methylreductase, and 5 μg of ^{63}Ni-labeled methylreductase. The center well contained 43.5 μg of mouse antimethylreductase serum. No precipitation was observed when 50 μg of control serum was substituted for antimethylreductase serum (data not shown).

mol of protein. These facts clearly show that the methylreductase is a nickel-containing enzyme and that the stoichiometry of nickel to F_{430} in the native protein is close to 1:1.

Although the methylreductase contained both nickel and F_{430}, it was possible that each was bound at a separate site on the enzyme. To clarify this issue, the chromophore from the nickel-labeled methylreductase was extracted with 80% boiling methanol and concentrated under a stream of nitrogen; its behavior was compared with that of F_{430} in HPLC and TLC. Approximately 75% of the label was extracted by this procedure.

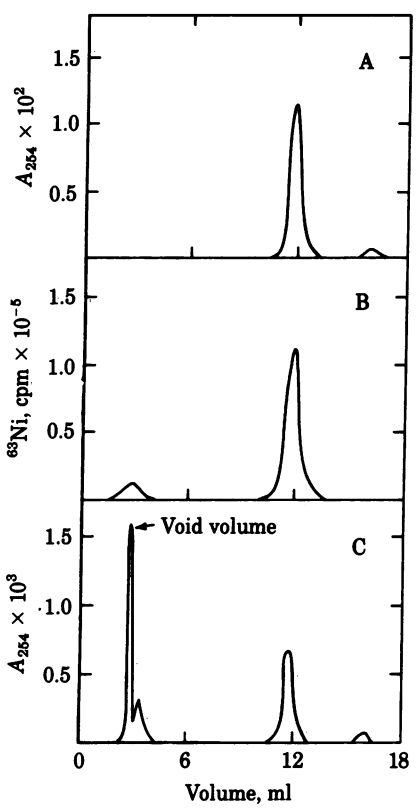

FIG. 4. HPLC of F_{430} and ^{63}Ni-labeled methylreductase chromophore. Columns were monitored for UV absorbance and for radioactivity. (*A*) Absorbance profile of a 1-μl sample of 0.01 M F_{430}. (*B* and *C*) Radioactivity and absorbance profiles, respectively, of a 25-μl sample of ^{63}Ni-labeled methylreductase chromophore (2×10^5 cpm); 71% of sample radioactivity was recovered after chromatography.

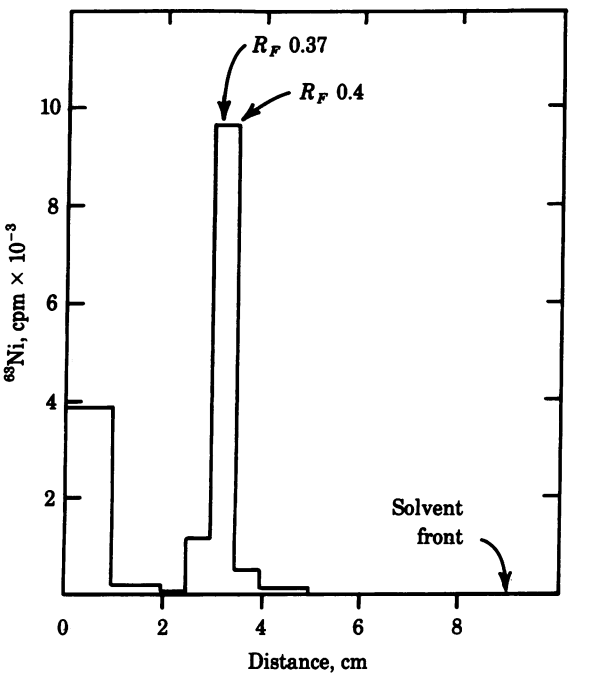

FIG. 5. TLC of F_{430} and ^{63}Ni-labeled chromophore on silica gel. Samples consisted of 2 μl of F_{430} and 2 μl of ^{63}Ni-labeled chromophore (16,000 cpm). The R_F of the chromophore was 0.37 and that of F_{430} was 0.4. The arrow indicates the solvent front. Approximately 96% of total applied radioactivity was recovered.

The HPLC elution pattern obtained with authentic F_{430} is shown in Fig. 4*A*, and that of the chromophore isolated from the ^{63}Ni-labeled methylreductase is shown in Fig. 4 *B* and *C*. More than 90% of recovered ^{63}Ni eluted in the identical position with authentic F_{430}. Furthermore, a corresponding peak was observed with the UV detector, and this fraction was yellow. Comparison of authentic F_{430} and the isolated chromophore from ^{63}Ni-labeled methylreductase by silica gel TLC is shown in Fig. 5. The nickel radioactivity and authentic F_{430} migrated with nearly identical mobilities in the solvent system used.

DISCUSSION

Our studies show that F_{430} is bound to the methylreductase of *M. thermoautotrophicum* at a ratio of 2 mol of F_{430} to 1 mol of protein. Considering the stoichiometry of subunits (α_2, β_2, γ_2) in the methylreductase (15), one F_{430} may be bound per trimer or F_{430} may be bound specifically to one type of subunit. The nature of the binding of F_{430} to the methylreductase is unclear; so far we have been able to quantitatively release F_{430} only under conditions that denature the enzyme complex (i.e., boiling methanol or $NaDodSO_4$ treatment); covalent attachment of F_{430} seems unlikely. The pivotal importance of F_{430} in the biochemistry of methane formation is becoming increasingly apparent. Results of ^{1}H NMR studies suggest the presence of either CH_3-S-coenzyme M or HS-coenzyme M in F_{430} (19). Evidence suggests that the compound is a tetrapyrrole, though it has not been established that it has a corrin type ring system (20). The chemical interaction of Ni and coenzyme M in methyl group reduction and possibly in CO_2 activation provide exciting possibilities for future study. It will be interesting to see whether F_{430} or analogous Ni-containing tetrapyrrole-like structures are involved in biological systems other than the methanogens.

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