Nickel-containing factor F₄₃₀: Chromophore of the methylreductase of *Methanobacterium*

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Contribution by R. S. Wolfe, March 15, 1982

**ABSTRACT** The yellow chromophore of the methyl-coenzyme M methylreductase of *Methanobacterium thermoautotrophicum* has been found to be the nickel-containing factor F₄₃₀. Treatment of ⁶⁴Ni-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 F₄₃₀, the stoichiometry being 1 mol of nickel per mol of F₄₃₀ and 2 mol of F₄₃₀ 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 dehydrogenase 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 (F₄₃₀) because of an absorption maximum at 430 nm. F₄₃₀ is nonfluorescent (10) and contains stoichiometric amounts of nickel (11, 12). Evidence showing the incorporation of δ-aminolevulinic acid and succinate into F₄₃₀ suggests that it is a tetrapyrole (13, 14), the first nickel-containing tetrapyrrole to be found in living organisms.

In spite of interest in F₄₃₀, 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 F₄₃₀ here, we identify this unique compound as the chromophore of the methylreductase.

**MATERIALS AND METHODS**

**Chemicals and Materials.** ⁶⁴NiCl₂ in 1 M HCl (11.1 Ci/g; 1 Ci = 3.7 × 10¹² 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 F₄₃₀ 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 ⁶⁴Ni and 32.8 μg of NiCl₂ was added. Nickel solutions were filter sterilized. Cultures were grown in 80% H₂/20% CO₂ 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 silica 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 F₄₃₀ as the Chromophore of the Methylreductase. Purified methylreductase from *M. thermoautotro-
**Protein-Bound Nickel in the Methylreductase.** Since F₄₃₀ 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 ⁶²Ni 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. thermotrophicum* on 31 μCi of ⁶²NiCl₂ 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 ⁶²Ni-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 ⁶²Ni-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

![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 (a). 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 F₄₃₀ of 21,000 M⁻¹·cm⁻¹ (12), was equal to 32 μM F₄₃₀ (---).](image1)

**Fig. 2.** Nondenaturing polyacrylamide gel electrophoresis of purified ⁶²Ni-labeled methylreductase. Samples containing 6.2 μg of purified ⁶²Ni-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.

**Table 2. Stoichiometry of nickel in methylreductase**

<table>
<thead>
<tr>
<th>Method of analysis</th>
<th>Protein, nmol/ml</th>
<th>Ni, nmol/ml</th>
<th>Ratio</th>
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<tbody>
<tr>
<td>Atomic absorption</td>
<td>31.7</td>
<td>60.5</td>
<td>1.91</td>
</tr>
<tr>
<td></td>
<td>31.7</td>
<td>62.1</td>
<td>1.96</td>
</tr>
<tr>
<td></td>
<td>60.0</td>
<td>113.0</td>
<td>1.88</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>1.92</td>
</tr>
</tbody>
</table>

**Purification of ⁶²Ni-labeled enzyme**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Protein, nmol/ml</th>
<th>Ni, nmol/ml</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparation 1</td>
<td>0.88</td>
<td>1.78</td>
<td>2.02</td>
</tr>
<tr>
<td>Preparation 2</td>
<td>0.29</td>
<td>0.58</td>
<td>2.00</td>
</tr>
<tr>
<td>Preparation 3</td>
<td>0.63</td>
<td>1.21</td>
<td>1.92</td>
</tr>
<tr>
<td>Preparation 4</td>
<td>1.33</td>
<td>2.75</td>
<td>2.07</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>2.00</td>
</tr>
</tbody>
</table>

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 H₂O and a 10-μl aliquot was used as the sample. A 2-μl sample of a 0.01 M solution of F₄₃₀ 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/ H₂O, 5:3:1:1 (vol/vol). HPLC was on a 0.39 × 30 cm C₁₈ μBondapak column, and the solvent was 30% methanol/1% formic acid adjusted to pH 3.0 with NH₄OH.

![Table 1. Chromatographic properties of methylreductase chromophore](image2)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Rₚ on TLC</th>
<th>HPLC retention time, min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>System 1</td>
<td>System 2</td>
</tr>
<tr>
<td>Chromophore</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanol extracted</td>
<td>0.80</td>
<td>0.33</td>
</tr>
<tr>
<td>After HPLC</td>
<td>0.81</td>
<td>—</td>
</tr>
<tr>
<td>F₄₃₀</td>
<td>0.61</td>
<td>0.34</td>
</tr>
</tbody>
</table>

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 H₂O and a 10-μl aliquot was used as the sample. A 2-μl sample of a 0.01 M solution of F₄₃₀ 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/ H₂O, 5:3:1:1 (vol/vol). HPLC was on a 0.39 × 30 cm C₁₈ μBondapak column, and the solvent was 30% methanol/1% formic acid adjusted to pH 3.0 with NH₄OH.
Our studies show that F430 is bound to the methylreductase of *M. thermoautotrophicum* at a ratio of 2 mol of F430 to 1 mol of protein. Considering the stoichiometry of subunits (α2, β2, γ2) in the methylreductase (15), one F430 may be bound per trimer or F430 may be bound specifically to one type of subunit. The nature of the binding of F430 to the methylreductase is unclear, so far we have been able to quantitatively release F430 only under conditions that denature the enzyme complex (i.e., boiling methanol or NaDodSO4 treatment); covalent attachment of F430 seems unlikely. The pivotal importance of F430 in the biochemistry of methane formation is becoming increasingly apparent. Results of 1H NMR studies suggest the presence of either CH3-S-coenzyme M or HS-coenzyme M in F430 (19). Evidence suggests that the compound is a tetrapyrole, 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 CO2 activation provide exciting possibilities for future study. It will be interesting to see whether F430 or analogous Ni-containing tetrapyrole-like structures are involved in biological systems other than the methanogens.

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**DISCUSSION**

mol of protein. These facts clearly show that the methylreductase is a nickel-containing enzyme and that the stoichiometry of nickel to F430 in the native protein is close to 1:1.

Although the methylreductase contained both nickel and F430, 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 90% boiling methanol and concentrated under a stream of nitrogen; its behavior was compared with that of F430 in HPLC and TLC. Approximately 75% of the label was extracted by this procedure.

The HPLC elution pattern obtained with authentic F430 is shown in Fig. 4A, and that of the chromophore isolated from the 63Ni-labeled methylreductase is shown in Fig. 4 B and C. More than 90% of recovered 63Ni eluted in the identical position with authentic F430. Furthermore, a corresponding peak was observed with the UV detector, and this fraction was yellow. Comparison of authentic F430 and the isolated chromophore from 63Ni-labeled methylreductase by silica gel TLC is shown in Fig. 5. The nickel radioactivity and authentic F430 migrated with nearly identical mobilities in the solvent system used.

**FIG. 5.** TLC of F430 and 63Ni-labeled chromophore on silica gel. Samples consisted of 2 μl of F430 and 2 μl of 63Ni-labeled chromophore (16,000 cpm). The *Rf* of the chromophore was 0.37 and that of F430 was 0.4. The arrow indicates the solvent front. Approximately 96% of total applied radioactivity was recovered.

**FIG. 4.** HPLC of F430 and 63Ni-labeled methylreductase chromophore. Columns were monitored for UV absorbance and for radioactivity. (A) Absorbance profile of a 1-μl sample of 0.01 M F430. (B and C) Radioactivity and absorbance profiles, respectively, of a 25-μl sample of 63Ni-labeled methylreductase chromophore (2 × 10^6 cpm); 71% of sample radioactivity was recovered after chromatography.
atomic absorption analysis. This investigation was supported by National Institutes of Health Grant AI 12277, National Science Foundation Grant PCM 78-25141, and U.S. Department of Energy Grant DOE-AC 02-80ER-10681.