Vol. 108, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 30, 1982 Pages 495-503

PRESENCE OF COENZYME M DERIVATIVES IN THE PROSTHETIC GROUP (COENZYME MF_{430}) OF METHYLCOENZYME M REDUCTASE FROM *METHANOBACTERIUM THERMOAUTOTROPHICUM*

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Received July 26, 1982

2-Mercaptoethanesulfonic acid (coenzyme M), or a derivative of it, and a yellow chromophore, known as the nickel-containing tetrapyrrole factor F_{430}, occur in the prosthetic group of methylcoenzyme M reductase in an equimolar amount, and bound to each other; this enzyme catalyzes the final step of methane production. The prosthetic group, which is called coenzyme MF_{430}, was isolated from the purified enzyme and was extracted from cells. The presence of coenzyme M was confirmed by a bioassay using *Methanobrevibacter ruminantium* and by the use of chemical and physicochemical analyses.

INTRODUCTION

Factors F_{430}, called after the characteristic absorption band at 430 nm (1), are yellow, non-fluorescing, nickel-containing compounds present in *Methanobacterium thermoautotrophicum* (1,2) and *Methanobacterium bryantii* (3). On the basis of labelling studies Thauer and his coworkers concluded that factors F_{430} are nickel tetrapyrroles structurally related either to sirohydrochlorin and sirohemes or to the corrinoid family (4,5,6). Recently, the structure of the chromophore modified by methylation was established by Pfaltz *et al.* (7).

Abbreviations used: TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; HS-CoM, 2-mercaptoethanesulfonic acid; CH_3S-CoM, 2-(methylthio)ethanesulfonic acid; $(S\text{-CoM})_2$, 2,2'-dithiodiethanesulfonic acid; $CoMF_{430}$, coenzyme MF_{430}.

0006-291X/82/180495-09$01.00/0

Previously, we reported that coenzyme M (HS-CoM, 2-mercaptoethanesulfonic acid) was present in some preparations of factor F_{430} (8). Coenzyme M and its methylated form, CH_3S-CoM, whose structures were elucidated by Taylor and Wolfe (9), are involved in the final step of methanogenesis (10):

$$CH_3S\text{-CoM} + 2H^+ + 2e \xrightarrow[\text{methylcoenzyme M reductase}]{} CH_4 + \text{HS-CoM}$$

Methylcoenzyme M reductase shows an absorbance maximum at about 430 nm (11) and the presence of factor F_{430} in the pure enzyme was recently established (12).

In this paper it will be demonstrated that about equimolar amounts of HS-CoM (or a derivative of it) and factor F_{430} are present in the reductase and that HS-CoM is present in factor F_{430} derivatives extracted and purified from *M. thermoautotrophicum*.

MATERIALS AND METHODS

Methylcoenzyme M reductase was isolated and purified to homogeneity from cells of *M. thermoautotrophicum* as previously described (11).

Factor F_{430} derivatives were obtained from *M. thermoautotrophicum* mass cultured in a medium containing 4.2 μM $NiCl_2$ as described by Doddema *et al.* (13). The derivatives were extracted from cells by anaerobic heating at 80°C with 40% aqueous ethanol. The subsequent purification, including ion-exchange column chromatography with QAE-Sephadex A-25 and DEAE-Sephadex G-25 and gelfiltration on Sephadex G-25 and Sephadex G-15, will be described elsewhere in detail. The compound, which appeared to be the most complete one, is called $CoMF_{430}$. Preparative thin-layer chromatography of $CoMF_{430}$ on Kieselgel-60 plates (0.50 mm, Merck) with 70% aqueous 2-propanol caused degradation of the compound and the concomitant formation of three chromophoric derivatives, viz. two yellow ones, $F_{430}A$ and $F_{430}B$, and one brown compound, called pMF_{430} (Table 1). By anaerobic acid hydrolysis (3 M HCl, 45 min, 110°C) of pMF_{430} and subsequent column chromatography a red product, called F_{red}, was obtained.

Alternatively, factor F_{430} derivatives were extracted from disrupted cells of *M. thermoautotrophicum* by treatment with $HClO_4$ at pH 2 and 0°C, and purified according to Diekert *et al.* (4). The procedure yielded one yellow derivative, called factor $F_{430}II$, and a red compound, called F_{560}.

Coenzyme M was quantitatively assayed by means of a bioassay with *Methanobrevibacter ruminantium* (14); the assay does not discriminate between HS-CoM, CH_3S-CoM and some other derivatives. To isolate the prosthetic group of methylcoenzyme M reductase the enzyme was precipitated under aerobic conditions with boiling 80% aqueous methanol and the protein pellet was washed with ice-cold 80% aqueous methanol. The pooled soluble fractions were concentrated under a stream of nitrogen gas and subsequently lyo-

philized. Aliquots of this material were applied to the culture medium of *M. ruminantium* before sterilization at 121°C.

Lyophilized samples of the pooled 80% methanol-soluble fractions were further purified by HPLC on a μBondapak C-18 column (0.78x 30 cm, Waters Assoc., Milford, Mass.). The flow rate was 2 ml/min, and the solvent was 100 mM ammonium acetate in 7.5% aqueous methanol. The material absorbing at 430 nm was collected, lyophilized and tested in the bioassay.

TLC was performed on plastic- or aluminum-backed Kieselgel-60 plates (0.25 mm, Merck). The solvents used were 1-butanol:acetic acid:water (4:1:1, v/v) (TLC-system I), and 70% aqueous 2-propanol (TLC-system II). In order to trace the presence or time-dependent emergence of HS-CoM (or a derivative) by TLC 25- or 50 μl samples of the various factor F_{430} derivatives (0.1-1 mg/ml) were hydrolyzed at 110°C with 0.05-3 M HCl in conically bottomed test tubes (10x0.6 cm). Immediately after addition of HCl the tubes were frozen in liquid nitrogen, then evacuated and fused, and subsequently subjected to acid hydrolysis. After hydrolysis the material was lyophilized to remove HCl, and the dried samples were dissolved in a minimal amount of distilled water and subjected to TLC. HS-CoM derivatives were stained by a spray of 0.1 M $AgNO_3$; then, the plates were dried and sprayed with 0.1 M $K_2Cr_2O_7$ in 1 M acetic acid: yellow (HS-CoM, $(S\text{-CoM})_2$) or orange (CH_3S-CoM) spots against a brown background were obtained. Less specifically, but in a more sensitive way, HS-CoM derivatives were stained by a spray of a freshly prepared mixture of equal amounts of $FeCl_3$ (2%, w/v in water) and potassium hexocyanoferrate (III) (1%, w/v in water); the plates were dried and subsequently sprayed with 2 M HCl. The procedure yielded blue spots against a white background.

The ^1H-NMR spectra of factor F_{430} derivatives were obtained with a Bruker WH90 spectrometer operating at 90 MHz and 25°C. ^2H_2O was used as solvent and the chemical shifts were calculated with respect to the H^2HO peak fixed at δ = 4.78 ppm: the position of the latter was determined previously with respect to internal 3-trimethylsilyl-(2,2,3,3-$^2H_4$)propionate under identical experimental conditions. All samples tested in ^2H_2O were lyophilized 3 to 4 times after repeated dissolution in ^2H_2O. Alternatively, the spectra of factor F_{430} derivatives in ^2H_2O were recorded with a Bruker WH360 spectrometer operating at 360 MHz and 30°C with the above-mentioned internal standard or with a Varian SC300 spectrometer operating at 300 MHz and 29°C with 2,2-dimethyl-2-silapentane-5-sulphonate as internal standard. Pulse Fourier transform mode was used in all instances.

RESULTS

Presence of coenzyme M derivatives in methylcoenzyme M reductase.

Pure methylcoenzyme M reductase was treated with boiling 80% aqueous methanol to dissolve the prosthetic group. The yellow supernatant reacted positively in the bioassay with *M. ruminantium*, developed to detect HS-CoM (or derivatives). Previous studies proved the presence of 2 mol factor F_{430} per mol pure enzyme (12). If one assumes that the molar absorbance of factor F_{430} in 80% methanol is equal to that in water (ϵ_{430} = 23x10^3 $M^{-1}cm^{-1}$) (2,3,5)

and if no other substances in the supernatant absorb at 430 nm then the molar ratio between the amounts of factor F_{430} and HS-CoM in the dissolved prosthetic group amounts to 1: 0.76 (standard deviation, $\pm$ 0.22; six bioassays with different amounts of the supernatant).

HS-CoM (or a derivative) remained associated with the yellow chromophore upon HPLC of the material obtained from the pure enzyme. The molar ratio between factor F_{430} and HS-CoM was determined to be 1 : 1.03 (s.d., $\pm$ 0.34; six bioassays) in the yellow fraction obtained in this way.

Presence of HS-CoM in factor F_{430} derivatives obtained from cells.

In the ^{1}H-NMR spectra of a number of factor F_{430} derivatives the presence of HS-CoM can be discerned easily. HS-CoM exposes a characteristic AA'BB' pattern situated at about δ = 3.0-3.2 (Fig. 1). The pertinent signals were absent in the ^{1}H-NMR spectra reported for $F_{430}M$, the methylated derivative which was investigated by Pfaltz *et al.* (7) and which lacks substituents attached to the chromophore. Fig. 1 represents the ^{1}H-NMR spectrum of pMF_{430}, a paramagnetic oxidation product of $CoMF_{430}$, that was obtained after preparative TLC of the latter on Kieselgel-60 plates. The paramagnetic character of pMF_{430} caused complete distortion of signals from protons attached to the chromophore; these signals are partly hidden by the noise level, but the recorded integral indicated a fairly large amount of protons in the regions δ = 1.5-3.0 ppm and δ = 3.5-4.5 ppm. The absence of well-defined additional signals, except for a doublet at δ= 1.38 ppm which will be discussed hereafter, excludes the presence of coenzyme M derivatives other than HS-CoM or $(S\text{-}CoM)_2$, an oxidation product of the former. In the 90 MHz ^{1}H-NMR spectrum of $CoMF_{430}$ the signals of HS-CoM are shifted upfield and the characteristic AA'BB' pattern of the CH_2-CH_2 group is absent

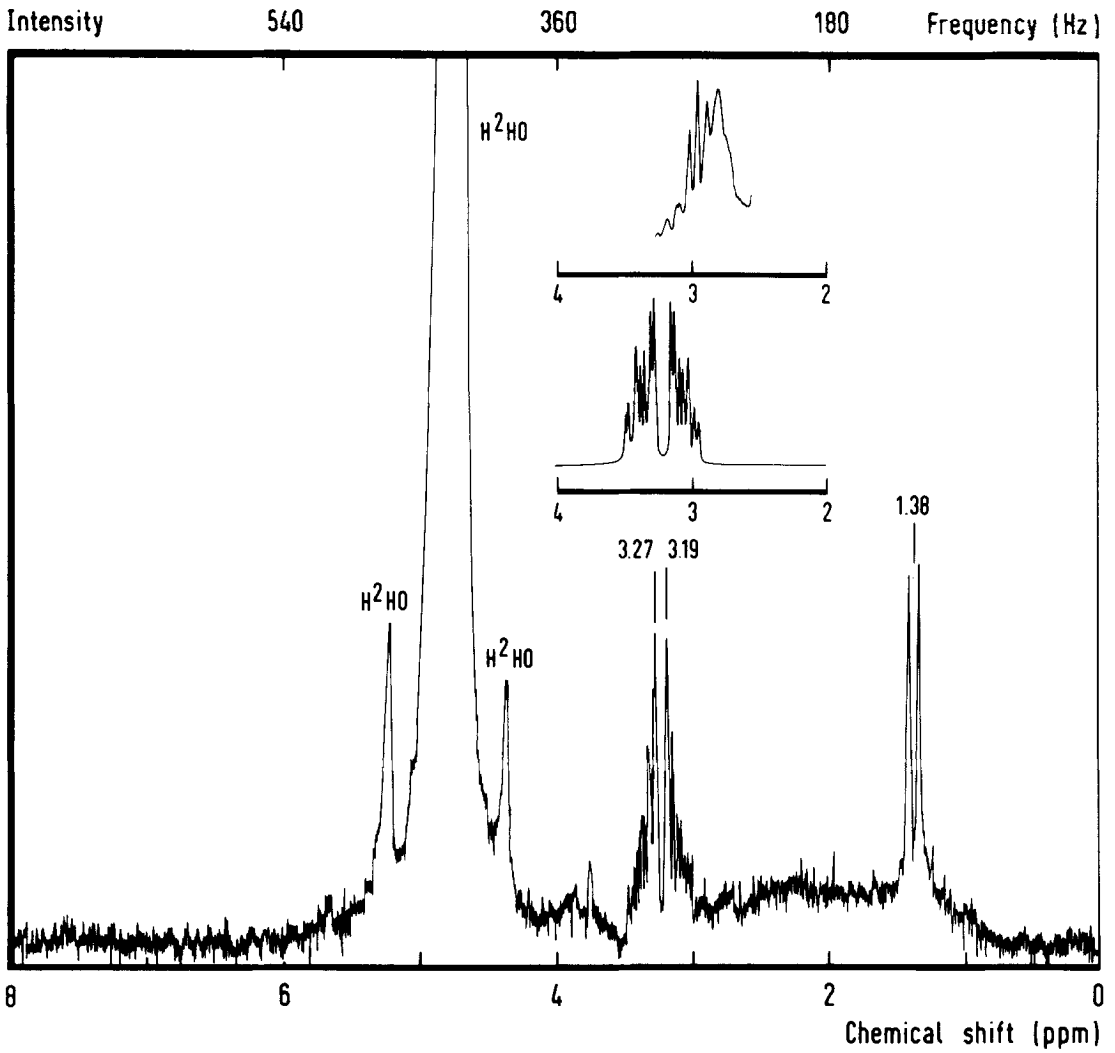

Fig. 1. 90 MHz ^{1}H-NMR spectra of pMF_{430}, $CoMF_{430}$, and HS-CoM. The lower spectrum was recorded with a solution of about 6.5 mg pMF_{430} in $^{2}H_2O$ at 25°C and p^{2}H 7.0; pulse width, 1.6 μs; number of scans, 7583. The middle spectrum was recorded in the region δ = 2-4 ppm with a solution of HS-CoM at p^{2}H 7.0 and 25°C. The top spectrum was obtained with a solution of $CoMF_{430}$ in $^{2}H_2O$ and p^{2}H 7.0 at 25°C; region δ = 2-4 ppm; pulse width, 5 μs; number of scans, 6920.

(Fig. 1). Presumably, both methylene groups take somewhat different positions with respect to each other in the conglomerate of $CoMF_{430}$.

The possibility that HS-CoM is a contaminant purified together with factor F_{430} derivatives was considered thoroughly but may be ruled out since the compounds behave quite differently during column chromatography and mixtures of the compounds were easily

Table 1. Properties of factor F_{430} derivatives.

	R_f-values		Presence[a] of	
	TLC-system I	TLC-system II	HS-CoM	X-CH(CH_3)-Y
$CoMF_{430}$	-[b]	-[b]	+	+
F_{430A}	0.21	0.49	-	+
F_{430B}	0.24	0.51	-	+
pMF_{430}	0.19	0.48	+	+
F_{red}	0.36	0.54	+	+
Factor F_{430II}	0.24	0.30	-	-
F_{560}	0.15	0.29	-	-
HS-CoM	0.55	0.90		
CH_3S-CoM	0.55	0.90		
$(S\text{-CoM})_2$	0.25	0.90		

[a] Presence (+) or absence (-) was derived from the ^{1}H-NMR spectra. The presence of HS-CoM was also checked by TLC after acid hydrolysis of the factor F_{430} derivatives.

[b] $CoMF_{430}$ degradates into F_{430A}, F_{430B} and pMF_{430}.

separated: factor F_{430} and HS-CoM derivatives show different R_f-values on the routinely used TLC systems (Table 1). Free HS-CoM was absent in chromatograms of the HS-CoM-containing species $CoMF_{430}$, pMF_{430} and F_{red}.

The liberation of HS-CoM from F_{430} derivatives upon acid hydrolysis was tested by TLC. HS-CoM was released by anaerobic treatment of $CoMF_{430}$ with 0.05 M HCl at 110°C for 5 min. HS-CoM was identified as described in Materials and Methods. The binding of HS-CoM in pMF_{430} is rather resistant towards acid hydrolysis, since F_{red}, a product obtained from pMF_{430} by anaerobic hydrolysis with 3 M HCl at 110°C for 45 min still contained HS-CoM in a bound form (Table 1). However, after prolonged acid hydrolysis of pMF_{430} free HS-CoM could be detected by TLC.

Preparative TLC, at which $CoMF_{430}$ was quite extensively exposed to oxygen and to (acid) Kieselgel for several hours, also caused the release of HS-CoM: as a consequence, the latter compound was

absent in the diamagnetic products $F_{430}A$ and $F_{430}B$ (Table 1). Factor $F_{430}II$ and F_{560}, which were extracted from disrupted cells by the use of $HClO_4$, did not contain HS-CoM either.

The proton ratios in the 300 MHz ^{1}H-NMR spectrum of $CoMF_{430}$ (not shown) revealed that the chromophore of $CoMF_{430}$ and HS-CoM occur in a 1:1 molar ratio. Besides the signals which could be attributed to HS-CoM a doublet was observed at δ = 1.38 ppm (3 H, J = 7.03 Hz, Fig. 1). The doublet is coupled with a quartet at δ = 4.21 ppm (1 H, J = 7.03 Hz). In Fig. 1 the signal is partly obscured by noise and the broadened H^2HO peak, but its presence was clearly discerned in the 360 MHz ^{1}H-NMR spectrum (not shown) and the coupling between the doublet and the quartet was demonstrated by means of selective decoupling performed in this spectrum. The position and the splitting pattern of the methine-proton point to a CH-group situated between two electron-withdrawing groups (X,Y). The X-CH(CH_3)-Y moiety was also present in a number of other factor F_{430} derivatives (Table 1). HS-CoM and the X-CH(CH_3)-Y moiety occur in equimolar amounts as judged on the basis of the proton ratios in those factor F_{430} derivatives which contain both groups.

DISCUSSION

By heating cells of *M. thermoautotrophicum* with 40% aqueous ethanol at 80°C a factor F_{430} derivative, called $CoMF_{430}$, was extracted that appeared to contain HS-CoM and the F_{430}-chromophore in a 1:1 molar ratio. Preparative TLC caused the degradation of $CoMF_{430}$ and the concomitant formation of three chromophoric compounds: two of them called $F_{430}A$ and $F_{430}B$, both lack HS-CoM, whereas a third product, the paramagnetic pMF_{430}, still contains HS-CoM. In pMF_{430} the binding between the chromophore and HS-CoM is more resistant towards acid hydrolysis as compared to $CoMF_{430}$. These findings suggest the presence of an additional and yet un-

resolved binding of HS-CoM formed during the conversion of the diamagnetic into the paramagnetic form of factor F_{430} derivatives. Extraction of cells of *M. thermoautotrophicum* with $HClO_4$ (pH 2, 0°C) caused the loss of HS-CoM from the chromophore of factor F_{430}. As a result, factor $F_{430}II$ and F_{560} are devoid of HS-CoM.

Previously (8), we reported the presence of methylcoenzyme M in a factor F_{430} derivative obtained from *M. thermoautotrophicum*. This result would imply that coenzyme M derivatives are attached to the chromophore most probably *via* the sulfonic acid group. Such a binding is consistent with the supposed role of the HS-CoM moiety in the prosthetic group of methylcoenzyme M reductase. The ^{1}H-NMR spectra of a number of factor F_{430} derivatives revealed the presence of HS-CoM and a X-CH(CH_3)-Y moiety in a 1:1 molar ratio; the latter moiety was also present in factor F_{430} derivatives that lack HS-CoM. This might indicate that the moiety functions as a spacer between HS-CoM and the chromophore of $CoMF_{430}$, thus interconnecting the acidic sulfonic acid group and possibly an acetyl or a propionyl residue of the tetrapyrrole chromophore. The X-CH(CH_3)-Y moiety was absent in factor $F_{430}II$ and F_{560}. By treatment of pure methylcoenzyme M reductase of *M. thermoautotrophicum* with boiling 80% aqueous methanol a supernatant was obtained which contained a factor F_{430} derivative and HS-CoM (or a derivative) in a molar ratio of about 1:0.76. HS-CoM remained associated with the factor F_{430} derivative upon HPLC and a molar ratio of 1:1 was found after this treatment. Ellefson *et al.* (12) reported recently that factor F_{430} could be extracted from methylcoenzyme M reductase by treatment with 80% aqueous methanol and they found two mol factor F_{430} present per mol enzyme. The results reported here present evidence that factor F_{430} and HS-CoM are bound to each other in what is now called coenzyme MF_{430} ($CoMF_{430}$). Probably two of these groups are present per molecule of enzyme.

Preliminary results indicate that $CoMF_{430}$ contains more molecular units attached to the chromophore besides the two described here (unpublished results).

Methylcoenzyme M reductase deals with HS-CoM derivatives in two ways: CH_3S-CoM is a substrate, and HS-CoM bound in $CoMF_{430}$ forms part of the prosthetic group.

ACKNOWLEDGEMENT

We thank Mr. Herman Brinkhof for kindly recording the 90 MHz and 360 MHz ^{1}H-NMR spectrum and Mr. Simon van Krimpen from TNO, Delft, for recording the 300 MHz ^{1}H-NMR spectrum of $CoMF_{430}$.

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