Sirohydrochlorin, a precursor of factor F_{430} biosynthesis in *Methanobacterium thermoautotrophicum*

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Factor F_{430} is a nickel-containing coenzyme of methanogenic bacteria with porphinoid structure which is derived from uroporphyrinogen III. It is shown that sirohydrochlorin is metabolized by cell free extracts of *Methanobacterium thermoautotrophicum* to factor F_{430} demonstrating that this compound, or a reduced form of it, is an intermediate in the biosynthesis of F_{430}, and not only of vitamin B_{12} and siroheme.

Factor F_{430} *Sirohydrochlorin* *Tetrapyrrole* *δ-Aminolevulinic acid* S-*Adenosylmethionine*
Methanobacterium thermoautotrophicum

1. INTRODUCTION

Factor F_{430}, a yellow non-fluorescent nickel-containing chromophore, is the coenzyme of the methylcoenzyme M reductase [1]. The structure of this compound has been previously shown to consist of an uroporphinoid (type III) ligand skeleton with an attached carbocyclic ring. The chromophore system is a tetrahydro derivative of the corphin system which combines the structural elements of both porphyrins and corrins. Such a system has not been previously encountered among natural porphinoids [2,3]. It has been shown that factor F_{430}, like all naturally occurring porphinoids (chlorophylls, hemes, cytochromes, sirohemes and corrinoids), is synthesized via uroporphyrinogen III (fig.1) [4]. It is also known that factor F_{430} contains 2 methyl groups derived from methionine [5] and that methanogenic bacteria contain corrinoids. From this information it has been suggested, that F_{430} is synthesised via sirohydrochlorin or a dihydrosirohydrochlorin.

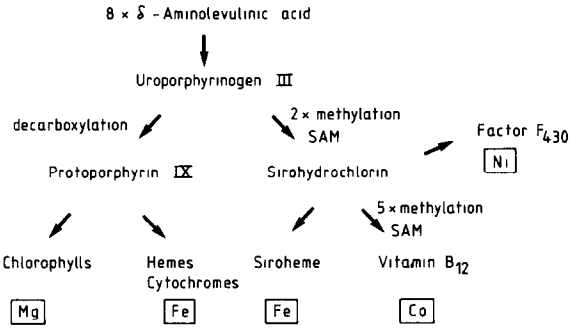

Fig.1. Biosynthetic pathways of natural tetrapyrroles.

Our intention was to investigate the role of sirohydrochlorin in the biosynthesis of F_{430} by studying the incorporation of labeled sirohydrochlorin (^3H, ^{14}C) into F_{430} by cell-free extracts of *Methanobacterium thermoautotrophicum*.

2. MATERIALS AND METHODS

2.1. *Culture conditions and preparation of cell free extracts*

M. thermoautotrophicum ΔH, DSM 1053, was grown in medium 1 described by Balch et al. [6]. This medium was supplemented with 6 mg/l $NiCl_2 \cdot 6H_2O$, which was omitted in the last scale-

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up step of the cultivation to suppress the feed-back inhibition of the F_{430} biosynthesis. The bacteria were cultivated either in 1 l bottles containing 200 ml medium or in a 20 l fermenter with H_2/CO_2 (80:20, v/v) as carbon and energy sources. After centrifugation and washing the cells were suspended in an incubation buffer (pH 6.8), containing 0.01 M potassium phosphate, 0.06 M $NaHCO_3$, 0.01 M $MgCl_2 \cdot 6H_2O$, 20 mg penicillin, 100 μg mercaptoethanesulfonate, 1 mg DNase (pancreas) from Merck (Darmstadt), and disrupted by 2 subsequent anaerobic passages through a French pressure cell. The cell debris was separated by centrifugation (Beckman JA-20 rotor, 18000 rpm, 5°C, 20 min) under an N_2 atmosphere and the supernatant (crude extract) was used for the studies.

2.2. *Metabolization of 5-aminolevulinic acid by crude extracts*

2 mg 5-amino[4-^{14}C]levulinic acid (ALA) (7860 Bq) were added to 40 ml crude extract. The reaction was performed in 120 ml serum vials (gas atmosphere H_2/CO_2 = 80:20 (v/v)) at 55°C at 120 rpm.

2.3. *Metabolization of sirohydrochlorin by crude extracts*

10.43 μmol [2,7-*methyl*-^{3}H, 4,5,9,10,14,15,16, 20-^{14}C]sirohydrochlorin were added to 44 ml crude extract. The specific radioactivity was 77.7 Bq/μmol consisting of 515 Bq ^{3}H and 295 Bq ^{14}C and in some experiments additionally 8300 Bq $^{63}NiCl_2$. Sirohydrochlorin was isolated and purified as the octamethyl ester [7]. The reaction was performed as described in section 2.2.

2.4. *Isolation of the metabolic products*

An equivalent volume of cold methanol (4°C) was added to the incubation mixture. After centrifugation (18000 rpm, 5°C, 20 min), the sediment was extracted with several batches of 20 ml methanol by heating for 1 h at 80°C. The extracts were recovered by evaporating the solvent, and then redissolved in 5 ml of 25 mM K-phosphate buffer (pH 7.0) and adsorbed on DEAE-Sephadex A-25 [8]. The column was washed with the same buffer and dried overnight. Thereafter, the bound metabolites were esterified (methanol/H_2SO_4 = 95:5 (v/v)) to yield the F_{430} methyl esters (F_{430} M). After 24 h the resulting methyl esters were extracted with chloroform. After evaporation of the chloroform, the residue was dissolved in 0.5 ml methanol, applied on silica gel 60 TLC plates (Merck) and developed with methanol/ethyl acetate/chloroform (1:2:3, v/v). The UV-Vis spectra and the radioactivities were measured. The fractions absorbing at 430 nm were separated by HPLC (RP-18) and eluted either with a gradient of 0.01 K-phosphate buffer, pH 6.8, and 80% methanol in water, as described by Livingston et al. [3], or with methanol. The absorbance of the eluate was recorded at 206 and 430 nm. The radioactivity of the fractions containing F_{430} M were measured with a beta-counter.

2.5. *Analytical methods*

The UV-Vis spectra were recorded on an HP 8450 A spectrophotometer. Molar extinction coefficients $E_{430}(H_2O)$ or 23100 cm^2/mmol and $E_{430}(CH_3OH)$ of 21900 cm^2/mmol [3] were taken as a basis for the calculations. The radioactivities were determined with a rackbeta II (LKB, Wallac) and the Unisolve 100 scintillator system (Zinsser, Frankfurt).

3. RESULTS AND DISCUSSION

Thauer and co-workers [2–5] used growing and resting cells for biosynthetic studies to produce factor F_{430} and analyze its structure. For the present experiments a cell-free system was prepared because it is unlikely that sirohydrochlorin is incorporated by intact cells. In a first experiment we proved the operability of the cell-free system and investigated the incorporation rate of ALA into factor F_{430}. The methylated factor (F_{430} M) was purified by subsequent TLC and HPLC chromatographical methods to approximately constant specific radioactivity. However, the working up and purification procedure was terminated after a second HPLC run because of appreciable losses of factor F_{430} (table 1). About 5% of added ALA was incorporated into F_{430}. After the second HPLC step, however, only 1.2% of the initial radioactivity applied as [^{14}C]ALA could be found in the F_{430} M fraction. This loss of radioactivity can be accounted for by the loss of sample during the working up procedure. The isolated F_{430} M did not contain any detectable impurity as confirmed

by UV-Vis, TLC and HPLC. As with [^{14}C]ALA, radioactivity was incorporated with either L-*S*-[*methyl*-^{3}H]adenosylmethionine (the methylating agent) or with ^{63}Ni (not shown).

Approximately the same amount (5%) of the labeled sirohydrochlorin was incorporated into F_{430} (table 2). The observed large decrease in specific radioactivity after the second purification step reflects the removal of other sirohydrochlorin-derived substances. After further purification steps (2 TLC and 2 HPLC runs) the specific radioactivities remained nearly constant. These data show that sirohydrochlorin, or a derivative of sirohydrochlorin (i.e. a reduced form) is incorporated into F_{430}.

To distinguish between the incorporation of sirohydrochlorin or a reduced form we studied the effect of crude extract on sirohydrochlorin with spectrophotometric methods. Fig.2 shows the time-dependent metabolisation of sirohydrochlorin by the crude enzyme extract of *M. thermoautotrophicum*. The reaction mixture containing cell-free extract, sirohydrochlorin and $NiCl_2$ (fig.2, plot 1) was incubated anaerobically. UV-Vis spectra were recorded as indicated. Sirohydrochlorin concentration decreased and a novel absorption band at 420 nm appeared. As sirohydrochlorin is a rather unstable compound, a control experiment was performed. The UV-Vis spectra demonstrate that sirohydrochlorin is degraded in the absence of the enzyme fraction within the incubation period. However, the redox state of the true biological intermediate could not be identified from these data, because no spectrophotometric data are available for reduced sirohydrochlorins.

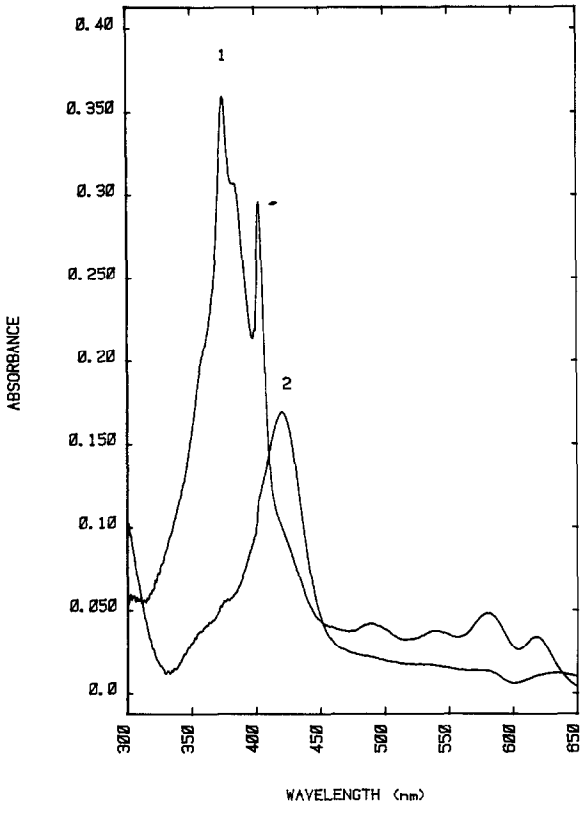

Fig.2. Difference spectra of anaerobic incubations of sirohydrochlorin in cell-free extracts of *M. thermoautotrophicum* after 5 min (plot 1) and 4 h (plot 2).

Our experiments show strong evidence that sirohydrochlorin, or a reduced form of it, is, as suggested by Gilles and Thauer [4], an intermediate of F_{430}-biosynthesis.

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