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REDUCTIVE ACTIVATION OF METHANOL: 5-HYDROXYBENZIMIDAZOLYLCOBAMIDE METHYLTRANSFERASE OF *METHANOSARCINA BARKERI*

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Methanol: 5-hydroxybenzimidazolylcobamide methyltransferase (MT_1) from *Methanosarcina barkeri*, which is one of the enzymes responsible for the transmethylation from methanol to coenzyme M, was found to be activated in the presence of hydrogenase and ferredoxin. This activation was shown to involve a reduction of the bound corrinoid to the Co (I) level, and was demonstrated by spectrophotometry and chemical conversion of reduced MT_1 to its methylated form. The reducing system of hydrogenase and ferredoxin was able to reduce dithiols, like dithiodiethanesulfonate and cystine to their monomers, in the presence of a corrinoid, which acts as an electron carrier. The ferredoxin was purified 133-fold and was tentatively identified on the basis of spectral properties and iron content of 3.8-4.0 atoms iron per molecule ferredoxin (12,000 daltons).

The pathway of methanogenic conversion of methanol by *Methanosarcina barkeri* was subject of various studies. A role of corrinoids was proposed after the discovery that Co-methyl-5,6-dimethylbenzimidazolylcobamide (methylcobalamin, CH_3-B_{12}-DMBI) could serve as a precursor for methane production [1]. It was demonstrated that methanol could be converted to CH_3-B_{12}-DMBI by extracts of *M. barkeri* in the presence of ATP, Mg^{2+} and H_2 [2]. Resolution of the enzymic system concerned demonstrated the involvement of a corrinoid protein, ferredoxin, a heat stable cofactor and an unknown protein [3]. Recently [4], it was found that the enzymic system which was responsible for the formation of 2-(methylthio)ethanesulfonate (CH_3S-CoM) from methanol consists of two enzymes, methanol: 5-hydroxybenzimidazolylcobamide (B_{12}-HBI) methyltransferase (MT_1) and Co-methyl-5-hydroxybenzimidazolylcobamide (CH_3-B_{12}-HBI): 2-mercaptoethanesulfonate (HS-CoM) methyltransferase (MT_2). MT_1 is a corrinoid-containing, oxygen-sensitive enzyme. The bound corrinoid, which contains 5-hydroxybenzimidazole (HBI) as α-ligand, was methylated by methanol in the presence of ATP [5]. MT_2 is insensitive to oxygen and transfers the methyl moiety from Co-methyl corrinoids to HS-CoM. The enzyme system, as measured in crude cell-free extracts, was subject to activation and inactivation [6]. Activation was performed by preincubation in the presence of H_2 and catalytic amounts of ATP.

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Another hydrogen-donating system, pyruvate dehydrogenase, could replace H_2 in the activation process [6].

Here we report the resolution of the system effective in the reductive activation of MT_1. Hydrogenase and ferredoxin (which was purified 133-fold) are shown to be involved. These compounds can bring about also a corrinoid-dependent reduction of oxidized thiols like dithiodiethanesulfonate $(S\text{-}CoM)_2$, cystine and dithiodiethanol.

MATERIALS AND METHODS

Methanosarcina barkeri, strain MS (DSM 800) was grown on methanol and harvested as described before [7]. Cell-free extracts were prepared in 10 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)/K^+ buffer, pH 7.2, [6] and stored at -20°C under N_2. All preparations of assay mixtures, purifications and the determination of free thiol groups were performed anaerobically. Resolution of cell-free extracts into components was achieved by DEAE-cellulose (Whatman, DE-52) chromatography [4]. MT_2 was purified as described before [4]. MT_1 was purified with successive DEAE-cellulose and QAE-A50 Sephadex chromatography (publication in preparation). The activity of MT_1 was tested in a reaction mixture containing 13 µg of MT_2 (specific activity 3 $\mu mol.min^{-1}.mg^{-1}$ of protein) as described before [4]. Ferredoxin was obtained from crude cell-free extracts by DEAE-cellulose chromatography and elution with 0.35-0.40 M NH_4Cl. Ferredoxin-containing fractions were pooled, concentrated with a PM-05 Amicon filter, then applied to a Sephadex G-100 column (120 x 2.6 cm) equilibrated with 10 mM TES/K^+ buffer, pH 7.2, and eluted (8 ml/h) with the same buffer. Fractions containing ferredoxin were pooled. Ferredoxin was tested in an assay mixture of 0.1 ml in 10-ml vials containing: 1 mM hydroxycobalamin ($HO\text{-}B_{12}$-DMBI); 20 mM $(S\text{-}CoM)_2$; 20 µl hydrogenase (22 µmol benzylviologen reduced.$min^{-1}.ml^{-1}$); 10 mM TES/K^+ buffer, pH 7.2, and an adequate amount of ferredoxin. The reaction mixture was placed under H_2-N_2 (50%:50%) and incubation was started at 37°C. At various time intervals a vial was placed on ice to stop the reaction, then the HS-CoM concentration was measured spectrophotometrically at 412 nm after anaerobic reaction with 2,2'-dinitro-5,5'-dithiodibenzoic acid (DNTB) [8]. Total and non-heme iron were determined with bathophenantroline and mercaptoacetic acid [9]. Hydrogenase and ferredoxin proteins were determined according to Lowry [10] using bovine serum albumin as standard. MT_1 and MT_2 proteins were determined with Coomassie brilliant blue G-250 [11]. Hydrogenase was obtained as a fraction of not-retained protein after DEAE-cellulose chromatography of cell-free extracts and was used without further purification. Hydrogenase activity was measured by its reduction of benzylviologen according to Doddema et al. [12]. HPLC analysis of corrinoids was performed as described before [5]. Quantification of corrinoids as $CN\text{-}B_{12}$ derivatives was performed according to Pol et al. [13].

RESULTS AND DISCUSSION

The purification procedure of ferredoxin is described in Materials and Methods. Ferredoxin was purified 133-fold (Table 1) and was tentatively identified on the basis of its spectral properties (Fig. 1; absorption maxima at 276 and 384 nm; A384/A276 = 0.67). The molecular weight of the ferredoxin was estimated to be 12,000 daltons by Sephadex-G50 gelfiltration. The total

Table 1. Purification of ferredoxin from *M. barkeri*

Purification step	Total Activity[1] (U)	Specific Activity (U/mg)	Purification Factor	Recovery (%)
Crude extract	224	0.066	-	100
DEAE-cellulose	154	2.1	31.8	63
Sephadex G-100	79	8.8	133.0	32

[1]Activity was measured in the presence of hydroxycobalamin (1.0 mM) and hydrogenase (0.44 µmol benzylviologen reduced.min^{-1}) as described in Materials and Methods and was expressed in units (U). One unit was equivalent to one µmol $(S\text{-}CoM)_2$ reduced per min.

iron content was determined to be 3.8-4.0 iron atoms per molecule ferredoxin as determined on the basis of a molar extinction coefficient reported for *Desulfovibrio gigas* Fd II (ε_{413} = 15,700 $m^{-1}cm^{-1}$) |14,15|. Heme-bound iron was absent.

In cell-free extracts of *M. barkeri* the methanol: HS-CoM methyltransferase system is known to be inactivated by various compounds, such as O_2, FAD, FMN

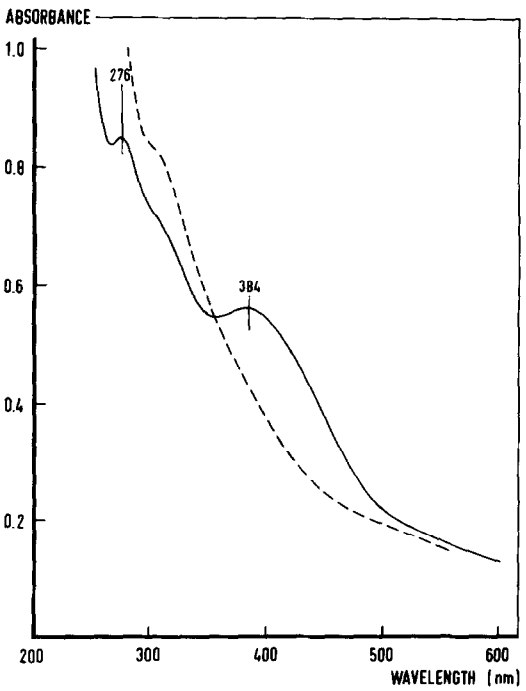


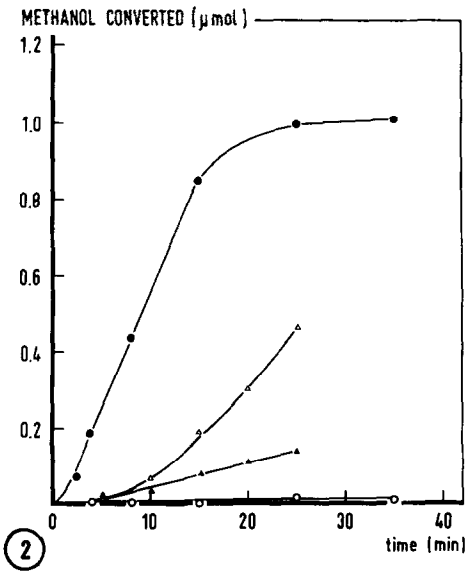
Fig. 1. Absorption spectra of purified ferredoxin from *M. barkeri*. The spectra were recorded in 10 mM TES buffer, pH 7.2, in anaerobic quartz cuvettes under 100% H_2 with a Cary 118 spectrophotometer; ———, purified ferredoxin; -----, purified ferredoxin reduced with sodium dithionite (0.02 mM).

and CH_3S-CoM, which act either as direct oxidizers or as electron acceptors in the presence of other components of the extracts. Activation of the system was achieved by preincubation under reductive conditions in the presence of ATP [6]. During purification of MT_1 a drastic decrease of activity was observed, which resulted in an almost inactive MT_1 after QAE-A50 Sephadex chromatography.

Reactivation of MT_1, obtained after DEAE-cellulose chromatography, which was previously exposed to air for 5 min, was performed in the presence of hydrogenase and ferredoxin (Fig. 2). The presence of only one of these compounds resulted in a small reactivation probably due to impurities in the preparations of MT_1 or hydrogenase.

Incubation of purified, inactive MT_1 together with hydrogenase and ferredoxin caused spectral changes (Fig. 3), which indicate a reduction of the bound corrinoid (absorption maximum at 472 nm) while a parallel increase of MT_1 activity was observed. The reduction procedure was further studied both with hydroxycobalamin and with MT_1 which contains a bound corrinoid. After reduction of hydroxycobalamin in the presence of hydrogenase and ferredoxin, the addition of methyliodide caused a decrease of absorption at 310 nm (Fig. 4). Both hydrogenase and ferredoxin were required in the process. The product formed was identified spectrophotometrically as methylcobalamin. These results are indicative for the formation of Co (I) corrinoids in the reduction process [16]. Incubation of inactive MT_1 in the presence of both hydrogenase and ferredoxin and addition of methyliodide resulted in the formation of CH_3-B_{12}-HBI, as judged by analysis of MT_1 by HPLC. In the absence of hydrogenase and ferredoxin no CH_3-B_{12}-HBI could be detected. These observations lead to the conclusion that hydrogenase together with ferredoxin is able to reduce free and protein-bound corrinoids to the Co (I) level.

The reduction of $(S\text{-CoM})_2$ to HS-CoM in cell-free extracts of *M. barkeri* incubated under H_2 was stimulated 24-fold upon addition of 1 mM hydroxycobalamin. This reduction could also be achieved in a mixture, containing hydrogenase, ferredoxin and hydroxycobalamin (Table 2). All three components were required in this process. The system reduced cystine and dithiodiethanol



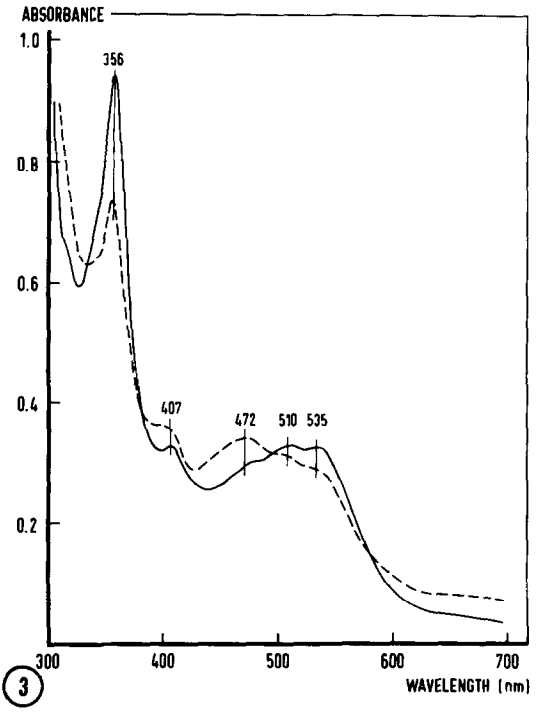


Fig. 2. Influence of hydrogenase and ferredoxin on the reductive activation of MT_1 (methanol: 5-hydroxybenzimidazolylcobamide methyltransferase) from *M. barkeri*. The activity of MT_1 (0.14 mg protein obtained after DEAE-cellulose chromatography and previously exposed to air for 5 min) was measured as given in Materials and Methods. 0.6 U MT_2 and extra additions: none (O); 0.022 U hydrogenase (Δ); 0.008 U ferredoxin (Δ); and 0.022 U hydrogenase plus 0.008 U ferredoxin (o). Incubations were performed under H_2-N_2 (50%:50%) at 37°C.

Fig. 3. Absorption spectra of MT (methanol: 5-hydroxybenzimidazolylcobamide methyltransferase) from *M. barkeri*. The spectra were recorded anaerobically in glass cuvettes under 100% H_2. ———, Inactive MT_1, obtained after QAE-A50 Sephadex chromatography; -----, MT_1 reactivated in the presence of ferredoxin (0.08 U) and hydrogenase (2.2 U).

at the same rate as $(S\text{-}CoM)_2$. B_{12}-HBI and B_{12}-DMBI derivatives were equally effective and reduction of $(S\text{-}CoM)_2$ proceeded faster if aquo- or hydroxy-cobalamins were applied as compared to cyanocobalamin (Table 2). The enzyme MT_1, which contains a firmly bound corrinoid, is also active in this corrinoid-dependent reduction of dithiols. The results demonstrate that both bound and free corrinoids are reduced by hydrogenase plus ferredoxin and function as electron carriers in the reduction process of dithiols.

The above results indicate that the reduction process which activates MT_1 requires the presence of hydrogenase and ferredoxin. Most probably the firmly bound corrinoid present in MT_1 is the target which requires reduction to the

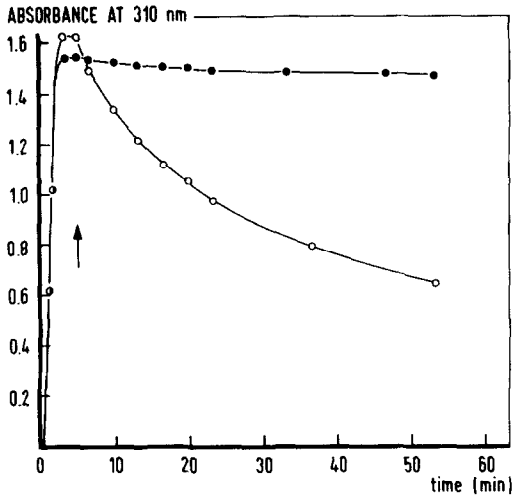


Fig. 4. Influence of ferredoxin on the reduction of hydroxycobalamin by hydrogenase of *M. barkeri*. The absorbance at 310 nm was measured in an anaerobic quartz cuvette containing a mixture (2 ml) of 0.1 mM HO-B_{12}-DMBI, 10 mM TES buffer, pH 7.2, and hydrogenase (2.2 U) (o). A second mixture contained also ferredoxin (0.08 U) (o). At the time indicated by an arrow 5 mM CH_3I was added. Reactions were carried out under 100% H_2 at 37°C.

Co (I) level. This was demonstrated by spectral changes of MT_1, by the chemical conversion of the reduced bound corrinoid into Co-methyl-5-hydroxybenzimidazolylcobamide in the presence of methyliodide, and by coupling the reduction

Table 2. Influence of corrinoids on the reduction of dithiodiethanesulfonate, $(S\text{-}CoM)_2$, in the presence of hydrogenase and ferredoxin from *M. barkeri*.

Corrinoid	Concentration (mM)	Activity (%)[1]
None	-	0
HO-B_{12}-DMBI[2]	1.0	100
HO-B_{12}-HBI	0.4	100
CN-B_{12}-DMBI	1.0	40
Ado-B_{12}-DMBI	1.0	60
MT_1[3]	0.015	10

[1]Activity was measured as indicated in Materials and Methods in the presence of hydrogenase (0.44 U) and ferredoxin (0.016 U).
[2]Activity in the presence of HO-B_{12}-DMBI was 0.014 µmol $(S\text{-}CoM)_2$ reduced.min^{-1} and was set at 100%.
[3]The corrinoid content of MT_1 was measured spectrophotometrically as CN-B_{12}-HBI(ε_{550} = 86.5 $mM^{-1}.cm^{-1}$).

process to the reduction of dithiols. The role of ATP in the activation process requires further study.

Two other methyl-transferring enzymes with firmly bound corrinoids are known: the "corrinoid" enzyme involved in the synthesis of acetate by *Clostridium thermoaceticum* |17| and 5-methyltetrahydrofolate: homocysteine methyltransferase, which is involved in methionine biosynthesis of many micro-organisms and animal liver. Reductive preactivation of the latter enzyme was reported before |18,19,20,21| and a similar process was suggested for the former enzyme |17|. Prereduction of the bound corrinoids appears to be a common prerequisite in the functioning of methyl-transfer.

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