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Ribonucleotide reductase in cell extracts of *Methanobacterium thermoautotrophicum*

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Cell free extracts of *Methanobacterium thermoautrophicum* were found to catalyze the reduction of CDP and GDP provided that the cells were harvested, disrupted and the extracts assayed under strictly anaerobic conditions. Ribonucleotide reduction is not affected by adenosylcobalamin and cell extracts do not catalyze the tritium exchange reaction between [5-$^3H_2$]adenosylcobalamin and water. The reduction of CDP requires ATP and that of GDP requires dTTP as positive allosteric effectors. In contrast, dATP at low concentration stimulates CDP reduction while at higher concentrations it inhibits the reductase reaction.

Adenosylcobalamin; Hydroxyurea; Ribonucleotide reductase; (Methanobacterium)

1. INTRODUCTION

Ribonucleotide reductases (EC 1.17.4) catalyze the irreversible reduction of the four common ribonucleoside 5′-phosphates to the corresponding 2′-deoxyribonucleoside 5′-phosphates, an essential and rate-controlling step in DNA synthesis and cell proliferation [1–3]. Thus far three types of reductases have been described. The non-heme-iron reductase first isolated from *Escherichia coli* contains 2 μ-oxo bridged antiferromagnetically coupled iron(III) ions and a tyrosyl radical as prosthetic groups. This type of reductase has also been demonstrated in *E. coli* infected with bacteriophage T_4, in green algae and in mammalian cells. Recently a manganese-containing ribonucleotide reductase has been isolated from *Brevibacterium ammoniagenes* and other coryneform bacteria and this enzyme probably also contains an organic radical [4]. Both these types of ribonucleotide reductases are inactivated by hydroxyurea or other hydroxylamine derivatives which reduce the organic radical. The third type of reductase is insensitive to hydroxyurea. It does not require a metal ion, but it has an absolute requirement for adenosylcobalamin as a coenzyme [5].

Thus far no obvious pattern for the distribution of these three enzyme systems is evident, although Dickman [6] has suggested that obligate anaerobic organisms use the adenosylcobalamin-dependent enzyme. Sprengel and Follmann [7] showed that a growing culture of the strictly anaerobic methanogen *Methanobacterium thermoautotrophicum* is able to incorporate cytidine into DNA without cleavage of the glycosidic bond, thus providing indirect evidence that the archaebacterium utilizes a ribonucleotide reductase system. However, cell-free extracts of this organism did not catalyze either the reduction of ribonucleotides or the tritium exchange reaction between [5-$^3H_2$]adenosylcobalamin and water. The exchange reaction is characteristic of the

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adenosylcobalamin-dependent reductases [8].

In the present paper we demonstrate that cell-free extracts of *M. thermoautotrophicum* strain Marburg do catalyze the reduction of ribonucleotides provided that the cells are harvested and disrupted, and the extracts are assayed under strictly anaerobic conditions. These extracts do not, however, catalyze tritium exchange between adenosylcobalamin and water.

2. MATERIALS AND METHODS

Radioactive nucleotides (spec. act. 15.2 Ci/mmol) were purchased from Amersham, Braunschweig. Unlabelled nucleotides, alkaline phosphatase (from calf intestine), and hydroxyurea were from Boehringer, Mannheim. [5′-$^3H_2$]Adenosylcobalamin was prepared as described [9]. *M. thermoautotrophicum* strain Marburg (DSM 2133) was from the Deutsche Sammlung von Mikroorganismen, Göttingen. Cultures were grown at 65°C in a glass fermenter containing 1.5 l mineral salts medium with 80% H_2, 20% CO_2 and 0.2% H_2S as described by Schönheit et al. [10]. The doubling time of the cells was between 90 and 120 min.

2.1. *Preparation of cell extracts*

All the following steps were performed at 4°C under strictly anaerobic conditions with hydrogen as the gas phase. Transfers were done in a glove box with an atmosphere of 95% N_2 and 5% H_2. Cells were harvested during mid-log phase (corresponding to an absorbance of 3.2 at 578 nm) by centrifugation (2700 × *g*, 30 min) and washed once in 50 ml of 0.1 M K-dimethylglutarate buffer, pH 7.3, containing 10 mM $MgCl_2$, 3 M glycerol, and 0.6 mg·l^{-1} resazurin (buffer A).

The cells were finally suspended in an equal volume of buffer A. Phenylmethylsulfonyl fluoride (50 μg·ml^{-1}) was added and an extract was prepared from the cell suspension by two passages through a French press pressure cell at 1100 psi. Streptomycin sulfate (1%) was then added and after incubation on ice for 30 min, the extract was centrifuged at 27000 × *g* for 45 min. The pellet was discarded. The cell-free extract was finally twice dialyzed against 500 ml of buffer A. Its protein content was determined using the Bio-Rad protein assay (Bio-Rad, München).

2.2. *Enzyme assays*

Assay mixtures containing 10 mM $MgCl_2$, 2 mM dithiothreitol, and nucleotides, as indicated below, in a total volume of 0.2 ml of 0.5 M K-dimethylglutarate buffer, pH 7.3, were pipetted into 8 ml serum bottles. The bottles were stoppered, evacuated for 1 min and filled with hydrogen gas at 1.2 bar for 1 min. This procedure was repeated 9 times. Cell extracts (0.3 ml, approx. 10 mg of protein) were added by injection through the septum and the bottles were incubated at the desired temperature. After 30 min the reaction mixtures were transferred to 1.5 ml Eppendorf tubes and heated at 100°C for 10 min. 20 μl of 0.5 M Tris-HCl buffer, pH 8.9, and 10 μl alkaline phosphatase (10 mg·ml^{-1}) were added and the tubes were incubated at 37°C for 90 min. The reaction mixtures were then heated at 100°C for 5 min, cooled and centrifuged. Deoxyribonucleosides were analyzed by HPLC as described [11].

For the tritium exchange experiments, [5′-$^3H_2$]adenosylcobalamin (0.07 mM, 9 × 10^6 cpm/μmol) was included in the reaction mixtures which were incubated in the dark, under H_2 at 55°C for 30 min. The mixtures were then frozen in liquid nitrogen. The water was collected by sublimation and the radioactivity of a 0.1 ml aliquot was determined by liquid scintillation counting in 10 ml Omniszintisol (Merck) cocktails.

3. RESULTS AND DISCUSSION

Ribonucleotide reductase activity has been measured for the first time in the methanogenic archaebacterium *M. thermoautotrophicum*. The results presented in table 1 demonstrate that a dialyzed cell extract prepared and incubated under strictly anaerobic conditions in the presence of H_2 is able to catalyze the reduction of cytidine-5′-diphosphate and of guanosine-5′-diphosphate. Under the assay conditions the cell extract generated sufficient reducing equivalents from molecular hydrogen and thus no added thiol, such as dithiothreitol, was needed. Adenosylcobalamin did neither stimulate nor inhibit CDP reduction. Indeed, cell extracts did not catalyze the tritium exchange between [5′-$^3H_2$]adenosylcobalamin and water. The reductase system of the strict anaerobe is very sensitive to oxygen. Incubation of an extract, prepared under H_2, in the presence of air for

Table 1

Reduction of ribonucleotides by dialyzed cell extracts of *M. thermoautotrophicum*

Assay conditions[a]	Effector nucleotide	Deoxyribonucleotide formation	
		nmol	%
CDP reduction	ATP (2 mM)	2.94[b]	100
	none	0.18	6
	dTTP (0.5 mM)	0.35	12
+ AdoCbl (40 μM)	ATP	2.88	98
+ hydroxyurea (20 mM)	ATP	1.21	41
+ hydroxyurea (100 mM)	ATP	0.41	14
+ air	ATP	0.62	21
GDP reduction	dTTP (0.5 mM)	1.64	100
	none	0.10	7
	ATP (2 mM)	0.18	11

[a] Assays contained 10 mM $MgCl_2$; 2 mM dithiothreitol; 50 μM [5-^{3}H]CDP or [8-^{3}H]GDP, respectively; 0.2 M K-dimethylglutarate buffer, pH 7.3; 12.6 mg protein; and effector nucleotides and other additions as indicated. The assay mixtures were incubated for 30 min at 55°C under a hydrogen atmosphere or where indicated in the presence of air

[b] From the growth rate, the size of the genome (10^9 Da) and the G-C content (48%) [12], it can be calculated that the rate of CDP reduction in vitro is approx. 8% of the deoxycytidylate formation required for DNA synthesis in vitro

30 min resulted in an 80% loss of activity. Even under anaerobic conditions ribonucleotide reduction proceeded linearly for only 10–15 min. It is thus not at all surprising that Sprengel and Follmann [7] were unable to detect enzyme activity in extracts from *M. thermoautotrophicum* harvested, disrupted, and assayed in the presence of air.

Ribonucleotide reductase of *M. thermoautotrophicum* is not very sensitive to hydroxyurea; even concentrations as high as 100 mM do not completely abolish activity. These results are in accord with our in vivo observations. The addition of 10 mM hydroxyurea to a growing culture does not affect the proliferation of the cells: concentrations as high as 50 or 100 mM are needed to inhibit growth.

The reduction of ribonucleoside diphosphates depends on the presence of nucleoside triphosphates as positive allosteric effectors. Thus, CDP reduction requires ATP at an optimum concentration of 1–2 mM (fig.1). The nucleotide dATP stimulates CDP reduction only at a much lower concentration (fig.2) and at higher concentrations it acts as an inhibitor. The reduction of GDP requires thymidine triphosphate as a positive effector, its optimum concentration is approx. 0.5 mM. The effects of these nucleotides on substrate reduction are specific. ATP does not

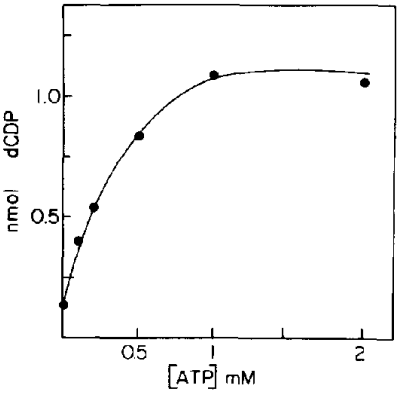

Fig.1. Dependence of CDP reduction on ATP. Assay conditions are as in table 1 with 7.8 mg protein.

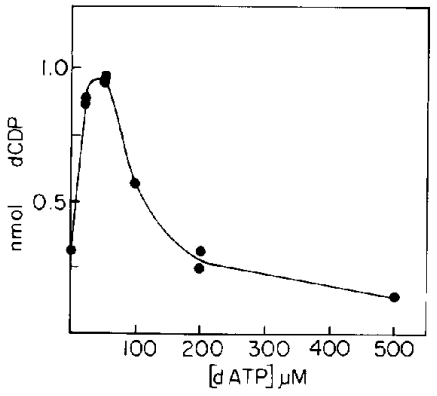

Fig.2. Effect of dATP on the reduction of CDP. Assay conditions are as in table 1 with 10.2 mg protein.

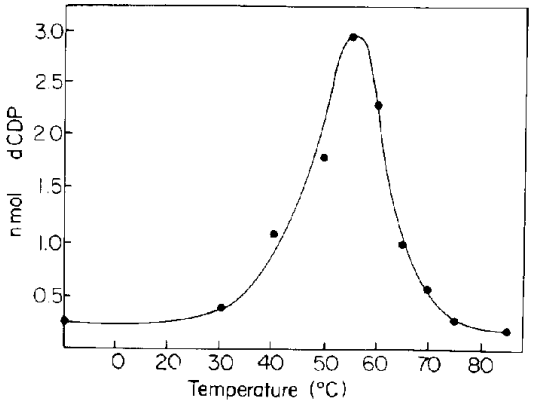

Fig.3. Temperature dependence of *M. thermoautotrophicum* ribonucleotide reductase in vitro. The assay mixtures described in table 1, containing 10.2 mg protein, were incubated for 30 min under a hydrogen atmosphere at the desired temperature.

stimulate GDP reduction and dTTP does not affect CDP reduction (table 1). This pattern of activity modulation is very similar to that observed in the non-heme-iron ribonucleotide reductases of *E. coli*, *Scenedesmus obliquus* and mammalian cells [1,11,13].

The temperature dependence of CDP reduction by cell extracts of *M. thermoautotrophicum* is shown in fig.3. The narrow optimum temperature range (55–60°C) observed in vitro is almost 10°C lower than the optimum temperature for the growth of the thermophile.

As mentioned above, three different types of ribonucleotide reductases are known. The enzyme of *M. thermoautotrophicum* does not require added adenosylcobalamin. However, this fact and the inability of cell extracts to catalyze the tritium exchange reaction do not provide conclusive evidence for an adenosylcobalamin-independent reductase. Recently, Kräutler et al. [14] have shown that the corrinoids of *M. thermoautotrophicum* are of the factor III type, containing 5-hydroxybenzimidazole instead of 5,6-dimethylbenzimidazole as the lower ligand. It is thus possible that in this organism adenosylfactor III is the only corrinoid that functions as a coenzyme for the reductase (but see [15]). On the other hand the allosteric behaviors of ATP and dATP on the reduction of CDP and that of dTTP on the reduction of GDP are very similar to the effects of the nucleotides in the adenosylcobalamin-independent reductases. It has been pointed out before that although the various ribonucleotide reductases differ in subunit structure and cofactor requirement, they all catalyze the reduction of ribonucleotides by a similar mechanism [2,16]. Because the enzyme from *M. thermoautotrophicum* is unique in its extreme sensitivity to oxygen, it may present yet another type differing in structure and catalytic site.

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