EVIDENCE FOR THE REDUCTIVE PATHWAY OF DEOXYRIBONUCLEOTIDE SYNTHESIS IN AN ARCHAEBACTERIUM

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1. Introduction

Enzymatic reduction of ribonucleotides is a requirement for the synthesis of DNA precursors and thus for DNA replication and cell proliferation in all bacteria and eukaryotic organisms previously studied. However, the ribonucleotide reductases (EC 1.17.4) and cofactors involved in that reaction exhibit very unusual structure variability in that they include non-heme-iron containing proteins, deoxyadenosylcobalamin (coenzyme B_{12}) requiring, or manganese-dependent enzymes [1–3]. No systematic pattern can as yet be recognized in the distribution of these various enzyme systems, although it has been suggested that anaerobic organisms generally have the B_{12}-dependent pathway [4]. Considering the exceptional phylogenetic position of the 'Archaebacteria' (methanogens, halophiles and thermoacidophiles) [5] and the paucity of information about their DNA synthesis it is of obvious interest to study the mode of deoxyribonucleotide formation in a representative species. We here show that DNA can, in fact, be efficiently labelled by a radioactive ribonucleoside precursor in *Methanobacterium thermoautotrophicum,* but that the process does not appear to involve coenzyme B_{12}.

2. Materials and methods

Radioactive nucleosides and nucleotides (spec. act. 10–20 Ci/mmol) were obtained from Amersham Buchler (Braunschweig). Deoxy-[5′-^{3}H]adenosylcobalamin (spec. act. 15 Ci/mol) was kindly provided by Dr H. P. C. Hogenkamp (Univ. Minnesota, Minneapolis). Lipoic acid was reduced with $NaBH_4$ and the product purified by distillation.

Cultures (250 ml) of *M. thermoautotrophicum,* strain Marburg, were grown at 65°C in a mineral medium under $H_2/CO_2/H_2S$ (80%, 20%, 0.2%) atmosphere [6]. The doubling time of the cells was 100 min. For preparation of cell-free extracts, bacteria from early or mid-log phase were ruptured in a French press and the homogenate centrifuged for 30 min at 30 000 × *g*. For DNA labelling experiments, a culture was grown for 8 h in the presence of 1 mCi (50–100 nmol) tritiated nucleoside or 0.25 mCi (50 nmol) [^{14}C]cytosine, respectively, yielding approximately 2 g packed cells.

Methanobacterium DNA was extracted in 5% SDS solution by the Marmur method [7], combined with passage of the cell suspension through a French press. DNA purification, formic acid hydrolysis (45 min at 170°C), and ion-exchange chromatography of liberated bases (Dowex 1 × 2 Cl^-, 0.2 M NH_4Cl (pH 10.6)) were done by standard procedures as in [8]. Each experiment was repeated 2 or 3 times and the results were always closely comparable.

Ribonucleotide reductase assays using tritiated ribonucleoside 5′-phosphates as substrates were performed under various conditions as described below. Bacterial protein (1–2 mg) was present in a total assay volume of 0.30 ml. In the available column-chromatographic substrate/product separation system as little as 0.1 pmol . mg^{-1} . min^{-1} formation of deoxyribonucleotide could have been detected [9].

Tritium exchange experiments contained, in a total volume of 0.50 ml dimethylglutarate buffer (pH 7.4), 50 μM deoxy-[5′-^{3}H]adenosylcobalamin, 25 mM dihydrolipoate or dithiothreitol, 1 mM nucleotides, and 1 mg bacterial protein (0.10 ml). Assays were incubated in the dark for 30 min after which time they were frozen in liquid nitrogen and radioactive water was collected by sublimation in vacuo.

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3. Results and discussion

Ribonucleotide reductases, because of their low intracellular concentration and limited stability, are notoriously difficult to determine in crude cell extracts. To avoid enzyme isolation from the strict anaerobes we chose an indirect in vivo assay of ribonucleotide reduction in which incorporation of a ribonucleoside into DNA is followed [8,10]. A growing culture of *M. thermoautotrophicum* was incubated with radioactive cytidine, the DNA was extracted and treated with DNase-free pancreatic ribonuclease under conditions known to degrade all contaminating RNA. The high M_r DNA was purified further on a hydroxylapatite column, and an aliquot subjected to base analysis. As seen in table 1, a significant proportion of the nucleoside (~1% of the total radioactivity present in the culture) was incorporated into the bacterial DNA and could be specifically located as cytosine after acid hydrolysis. In contrast, virtually no specific DNA labeling occurred when the free pyrimidine base, cytosine, was added to the culture medium. In control experiments the corresponding deoxyribonucleoside, 2′-deoxycytidine, led to the expected higher incorporation into DNA whereas thymidine is obviously not a good DNA precursor in this *Methanobacterium* species. (In a preliminary experiment we failed to detect thymidine kinase activity in cell extracts.)

Although uptake, utilization and salvage of nucleosides and bases have to our knowledge not been studied in detail in methanogenic bacteria, the results in table 1 appear clear-cut, and convincing evidence for direct, reductive ribonucleotide-to-deoxyribonucleotide conversion in *Methanobacterium*. As the reaction has been demonstrated before in several other bacteria and cyanobacteria, in animals and plants, it may now be concluded that the sequence:

purine, pyrimidine biosynthesis → ribonucleotides → deoxyribonucleotides → DNA

operates in all organisms.

The above in vivo experiments could not establish a specific cofactor requirement:

(i) The known reducing enzymes employ different transition metal (Mn, Fe, Co) complexes for catalytic activity [3];

(ii) On the other hand the methanogens contain and need unusual cofactors and metal ions [11].

Using a most sensitive assay system [9] with radioactive adenosine, cytidine and guanosine 5′-mono, di- or triphosphates as substrates we sought to measure ribonucleotide reductase activity in *M. thermoautotrophicum* extracts in vitro. Although the type and concentration of reductants (reduced lipoate, dithiothreitol, NADH, NADPH), of effector nucleotides (ATP, dATP, dGTP, dTTP), potentially catalytic metals (Mg^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, molybdate) and deoxyadenosylcobalamin, and the incubation temperatures (30–65°C) have been widely varied it was not possible to demonstrate in vitro deoxyribonucleotide formation. However, these unsuccessfull assays do not prove the absence of such an enzyme system; for example, it can have easily escaped detection if the anaerobic bacteria possess a very oxidation-sensitive protein and/or unknown cofactor which were inactivated during work-up.

Involvement of deoxyadenosylcobalamin (coenzyme B_{12}) in ribonucleotide reduction can be independently tested in the absence of substrate reduction and even in crude extracts if one measures the tritium exchange between deoxy-[5′-^{3}H]adenosylcobalamin and water. The nucleotide- and reductant-dependent reaction has proven to be a reliable indicator in all organisms containing a *Lactobacillus*-type ribonucleo-

Table 1
Labeling of *Methanobacterium* DNA by radioactive precursors

Precursor	Spec. act. purified DNA (dpm/A_{260} unit)	Total radioactivity of fractionated bases (dpm)
[5′-^{3}H]Cytidine	19 400	Cytosine, 79 400 Guanine, 1500
[2-^{14}C]Cytosine	200	–
Deoxy-[5′-^{3}H]Cytidine	34 000	Cytosine, 136 800 Guanine, 15 100
[*methyl*-^{3}H]Thymidine	2650	Thymine, 900

Table 2
Tritium release from deoxy-[5'-^{3}H]adenosylcobalamin catalyzed by bacterial extracts

Components of assay	Radioactivity of water (dpm/0.1 ml)
Lipoate $(SH)_2$, nucleotides	310
Dithriothreitol, nucleotides	1100
Methanobacterium extract	
+ lipoate $(SH)_2$, nucleotides	500
+ dithiothreitol, nucleotides	2100
Same extract, boiled 5 min	
+ lipoate $(SH)_2$, nucleotides	1400
+ dithiothreitol, nucleotides	2100
Lactobacillus extract	
– lipoate $(SH)_2$, nucleotides	720
+ lipoate $(SH)_2$, nucleotides	23 600
complete, + *Methanobacterium* extract	20 500
complete, + boiled *Methanobacterium* extract	21 400
Lactobacillus extract, boiled 5 min	460

ATP + dGTP or GTP + dTTP were present as nucleotides, incubation was at 37°C or 60°C

tide reductase [12,13], including *Clostridium* and anaerobically-grown *Corynebacterium* cells where the enzyme has also been purified and characterized. We have performed systematic assays with *Methanobacterium* and *Lactobacillus* extracts (table 2). *Methanobacterium* extracts, again tested under a wide variety of conditions, do not release radioactivity from tritiated coenzyme B_{12} in excess of the (non-enzymatic) blank values. They also do not significantly interfere with the tritium exchange catalyzed by *Lactobacillus* enzyme. Thus, although methanogenic bacteria contain corrinoid coenzymes [14], B_{12} catalysis must be denied for ribonucleotide reduction in *M. thermoautotrophicum*. This fact is yet another biochemical detail separating the methanogenic from other bacteria [11]. It is in accord with the postulated polyphyletic origin of ribonucleotide reductases [3], but not with an obligatory role of the B_{12} enzymes in anaerobes [4].

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