Deoxyribonucleotide Synthesis in an *Escherichia coli* Mutant (H 1491) which Lacks Ribonucleotide Reductase Subunit B2

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Z. Naturforsch. **44c**, 715–718 (1989); received February 24, 1989

Anaerobiosis, Deoxyribonucleotides, Deoxyribosyl Transfer, *Escherichia coli*, Nucleoside Phosphorylases, Ribonucleotide Reductase

An iron-sensitive mutant of *E. coli* with a Mudl phage insertion in the *nrdB* gene lacks subunit B2 of the key enzyme of DNA synthesis, ribonucleotide reductase. Nevertheless, these cells are capable of growing in minimal media under anaerobic conditions, indicating a second enzyme or pathway for deoxyribonucleotide synthesis. We here show that ribonucleotide reduction cannot be unambiguously measured in bacterial extracts whereas phosphorylase-catalyzed deoxyribosyl transfer does occur; however these salvage reactions could not function *in vivo* in the absence of deoxyribosides. It is suggested that the cells possess a specific, anaerobic ribonucleotide reductase which escapes detection under aerobic standard conditions, similar to the situation found in strictly anaerobic methanogens.

Introduction

Formation of the 2′-deoxyribonucleotides required for DNA replication is catalyzed by three different ribonucleotide reductase (EC 1.17.4) systems which either utilize deoxyadenosylcobalamin [1] or an organic free radical associated with protein-bound manganese [2] or binuclear iron centers [3] for catalysis of the hydrogen transfer reaction. The latter type of enzyme, which is found in all eukaryotes and in the gut bacterium *Escherichia coli*, is activated in the presence of oxygen which oxidizes a specific tyrosine residue of the smaller subunit 2 (Tyr-122 in *E. coli* subunit B2 [4]) in a spontaneous or enzyme-catalyzed [5, 6] reaction and thus generates the essential free radical. Hydroxyurea, a powerful radical scavenger, inhibits the iron-containing ribonucleotide reductases *via* reduction of the tyrosyl radical.

The O_2 dependence of deoxyribonucleotide synthesis in *E. coli* presents an obvious physiological and biochemical problem because the organism is a facultative anaerobe. This question became recently apparent when Hantke isolated an iron-sensitive mutant of *E. coli* K-12 with a Mudl phage insertion in the *nrdB* gene encoding ribonucleotide reductase subunit B2 [7]. It was observed that these bacteria would grow in minimal media under anaerobic (but not under aerobic) conditions, and were resistant to hydroxyurea.

Most anaerobic microorganisms possess adenosylcobalamin-dependent ribonucleotide reductases which do not require oxygen, but an involvement of corrinoid coenzymes in deoxyribotide synthesis has never been observed in *E. coli* [8, 10]. More importantly, the possible existence of two different ribonucleotide reductases in the same organism which might function under different growth conditions, although conceivable [9], has been considered unlikely and has not been addressed experimentally until recently [10, 11]. It was therefore of general interest to characterize deoxyribonucleotide synthesis in extracts of the *nrdB*-defective *E. coli* mutant cells in more detail.

Experimental

Materials and methods

Biochemicals and other reagents were purchased from Merck, Serva, or Boehringer, and radioactive nucleotides from Amersham-Buchler. Ribonucleotide reductase from *E. coli* B3 was prepared by the published procedure [12]. Protein was determined by the method of Bradford [13]. SDS-polyacrylamide gel electrophoresis was carried out in a Tricine buffer system [14]. Immunoblotting [15] was done with a B1–B2 antiserum kindly provided by Dr. B.-M. Sjöberg, University of Stockholm, and peroxidase-conjugated *anti*-rabbit IgG obtained from Dakopatts, Hamburg.

Deoxyribonucleotide formation was determined in a ribonucleotide reductase assay system [2] which involves dephosphorylation of all the substrate, effector, and product nucleotides by alkaline phosphatase after the incubation period, and separation of ribo- and deoxyribonucleosides, and free bases (if any) by

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/89/0700–0715 $ 01.30/0

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automated HPLC on an Aminex A9 ion exchange column. Unless stated otherwise, assays contained 0.1 mM (2 μCi) radioactive substrate, 2 mM Mg-acetate, 2 mM dithiothreitol, and 200 μg bacterial protein in a total volume of 50 μl 25 mM Hepes buffer, pH 7.5; they were incubated for 30 min at 30 °C.

Bacterial extracts

E. coli K-12 H1491 was grown as previously described [7] in medium E [16] containing 10 g K_2HPO_4, 3.5 g $Na(NH_4)HPO_4 \times 4\ H_2O$, 0.2 g $MgSO_4 \times 7\ H_2O$, 4 g glucose, 2 g citric acid monohydrate, 0.1 g tyrosine, 0.1 g tryptophane, 0.1 g phenylalanine, 5.4 mg *p*-aminobenzoic acid, 5.4 mg *p*-hydroxybenzoic acid, and 1 mg thiamine per l. Cells were cultivated under anaerobic conditions at 30 °C and harvested at about 0.3 mg dry weight per ml (wet weight, 1.2 g l^{-1}), and were stored at −20 °C.

The cells (5 g) were suspended in 7.5 ml 0.1 M Hepes buffer, pH 7.5, containing 2 mM dithiothreitol, and were broken in a Branson sonifier B-12 at 60% maximum power for 15 × 20 sec. After brief treatment in a glass homogenizer cell debris was removed by centrifugation at 30,000 × *g*, and the supernatant was centrifuged further for 90 min at 130,000 × *g*. A saturated ammonium sulfate solution was added to the clear supernatant to 30% saturation, and after 30 min the precipitate was removed by centrifugation. The $(NH_4)_2SO_4$ concentration was raised to 60%, and the resulting pellet was recovered and dissolved in 25 mM Hepes buffer, pH 7.5, containing 2 mM dithiothreitol.

The protein solution was fractionated on the Superose 12 (HR 10/30) column of a Pharmacia FPLC system, using 50 mM Hepes buffer, pH 7.5, containing 2 mM dithiothreitol at a flow rate of 0.8 ml min^{-1}. The eluate was collected in saturated ammonium sulfate solution and the protein precipitate was recovered by centrifugation. The samples were redissolved in and dialyzed against the above 25 mM Hepes buffer for 4 h, and were then used for enzyme assays.

Results and Discussion

Anaerobic cultures of *E. coli* H1491 were grown in a minimal medium not containing deoxyribose derivatives. Because these mutant cells are defective in the *nrdB* (ribonucleotide reductase protein B2) gene they should produce deoxyribonucleotides by an alternate ribonucleotide reductase, or by more indirect pathways. Nevertheless it was desirable to rule out the possibility of continued presence of the regular *E. coli* ribonucleotide B1−B2 complex. Protein fractions of the mutant cells were therefore probed with a B1−B2 antiserum. Indeed, Western blots exhibited no trace of monomeric protein B2 (M_r = 43,500) but they did show proteins in the range of monomeric subunit B1 (M_r = 87,000).

It was not possible to directly determine deoxyribonucleotide formation in cell-free extracts of *E. coli* H1491 by means of standard ribonucleotide reductase assays with radioactive CDP as substrate. These extracts, in contrast to controls prepared from *E. coli* B3 cells, catalyzed extensive formation of the free bases, cytosine and uracil, in addition to small amounts of deoxycytidine, precluding reliable estimation of pyrimidine deoxyribonucleosides in our chromatographic system [2]. Using GDP as substrate, 2′-deoxyguanosine formation could be measured in the same extracts because this product is unambiguously identified even in the presence of excess guanine. However, as the N-glycosidase (nucleosidase) activity by far exceeded deoxyriboside formation, further characterization of the latter process still required N-glycosidase-free enzyme preparations.

An efficient and rapid fractionation of bacterial proteins was achieved by chromatography on an FPLC Superose-12 gel filtration column (Fig. 1). The enzyme(s) catalyzing base liberation eluted first and were clearly separated from a fraction catalyzing deoxyriboside formation with only negligible accumulation of guanine; this fraction contained proteins of the 50,000 to 90,000 molecular weight range. Specific activities of up to 5 nmol deoxyguanosine · $h^{-1} \cdot mg^{-1}$ were observed in the presence of 1 mM GDP as substrate, 2 mM Mg^{2+}, and 1 mM dTTP, which is the common positive effector of GDP reduction. Product formation was not affected by up to 20 mM hydroxyurea, which inhibited ribonucleotide reduction over 90% in an enzyme of similar activity prepared from *E. coli* B3 cells. Neither the inclusion of adenosylcobalamin (40 μM), nor illumination of assay mixtures for photolysis of endogenous coenzyme B12 had any effects.

It is difficult to identify an unknown ribonucleotide reductase in partially purified cell extracts because of the great complexity and vulnerability of

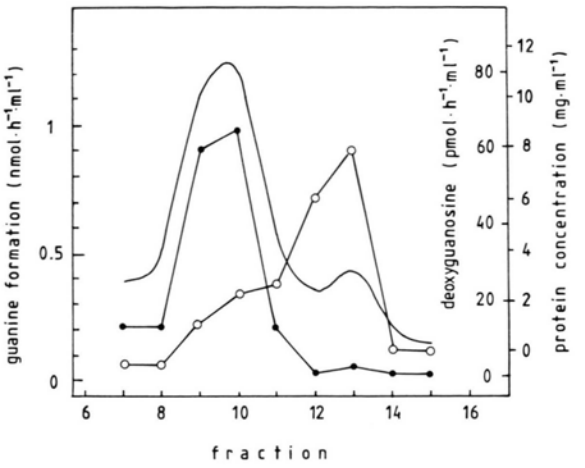

Fig. 1. Fractionation of an *E. coli* H1491 protein extract on a Superose-12 FPLC column. Drawn line: protein elution profile. (●): [^{3}H]Guanine formation from [8-^{3}H]GDP; (○): [^{3}H]deoxyguanosine formation from [8-^{3}H]GDP. *Cf.* Experimental section for further details.

these enzymes, but their dependence on dithiothreitol as *in vitro* reductant, comparable CDP and GDP reduction rates, and the universal allosteric effector specificities [9] can usually serve as guides. The enzyme fraction described above did not meet all these criteria (Table I). Thus, deoxyguanosine formation was only 10% reduced in the absence of dithiothreitol; deoxycytidine formation from CDP or CTP remained low and did not respond to ATP or dATP as effector nucleotides; and the reaction of GDP was also stimulated by dGTP which is unknown as positive effector for this substrate.

The experiments summarized in Table II provide an explanation. It is seen that deoxyguanosine is formed more efficiently from guanosine than from GDP, and that any deoxyribonucleoside together with phosphate ions is far more efficient in product formation than dTTP or dGTP; in contrast, ATP was totally inactive. These data indicate that the [^{3}H]gua-

Table I. Deoxyribonucleoside formation in partially purified protein fractions from *E. coli* H1491, analyzed in a ribonucleotide reductase assay system (*cf.* Experimental).

Substrate	Effector nucleotide	Assay composition	Rel. activity [%]
0.1 mM [8-^{3}H]GDP	none	complete	6
	1 mM dTTP	complete	100[a]
	1 mM dGTP	complete	93
	1 mM ATP	complete	4
	1 mM dTTP	Mg^{2+} omitted	4
	1 mM dTTP	dithiothreitol omitted	90
0.1 mM [5-^{3}H]CDP	none	complete	100[b]
	1 mM ATP	complete	70
	50–100 μM dATP	complete	68

[a] 350 pmol deoxyguanosine·h^{-1} formed in a standard assay.
[b] 130 pmol deoxycytidine·h^{-1}.

Table II. Dependence of deoxyguanosine formation from [8-^{3}H]-guanosine on a deoxyribose source and phosphate. Assays were performed in analogy to ribonucleotide reductase assays (*cf.* Experimental).

Substrates		Products	
Deoxyriboside [1 mM]	Phosphate [mM]	Deoxyguanosine [pmol h^{-1}]	Guanine [pmol h^{-1}]
None	0.2	8	14
Deoxyuridine	0.5	440	51
5-Fluorodeoxyuridine	0.5	410	49
Deoxycytidine	0.5	440	54
Thymidine	0.5	370	41
Thymidine	1.0	1500	
dTTP	1.0	845	400
Thymidine	5.0	3600	

nine-labeled deoxyguanosine obtained from [8-^{3}H]-guanosine(diphosphate) in presence of the enzyme fraction from *E. coli* H1491 *in vitro* is produced predominantly *via* deoxyribosyl transfer reactions on the nucleoside level, and not by ribonucleotide reduction. The dependence on phosphate and the limited accumulation of free base make purine nucleoside phosphorylase (*deoD* product; EC 2.4.2.1) and thymidine phosphorylase (*deoA* product; EC 2.4.2.4) likely candidates for the catalysis of deoxyriboside formation in the assay mixtures described above. These catabolic enzymes have been well characterized in enteric bacteria [17]. Nucleotides could serve as substrates because the enzyme fraction contained high phosphatase activity (data not shown).

However, salvage reactions can hardly provide all the deoxyribonucleotides required for growth of *E. coli* in deoxyribose-free medium, and our results do not rule out the existence of an alternate (hydroxyurea-insensitive, B12-independent) ribonucleotide reductase. Such an enzyme may be responsible for the 1–2% deoxyguanosine formed besides guanine in the first, larger M_r protein peak of the gel filtration shown in Fig. 1, but the small amount of product did not permit further characterization of that activity. It is also conceivable that an anaerobic reductase present in anaerobic cultures of the *nrdB*-defective mutant cells, or an activating system required for radical generation, became inactivated in air and thus escaped detection. We have encountered the latter type of enzyme in extracts of the strict anaerobe, *Methanobacterium thermoautotrophicum* [18], and a similar situation appears to exist in oxygen-sensitive *E. coli* strains (Oxy^s-11) [11]. On the other hand, ribonucleotide reduction was directly measurable in extracts of *E. coli* KK535, a strain carrying mutations in *(inter alia) nrdA, nrdB,* and the *deo* operon [10]; these cells are, therefore, much more suitable than H1491 for analysis of a second pathway of deoxyribotide biosynthesis because they lack purine nucleoside-degrading activities. Better understanding of all the variances found in different bacterial strains has to await purification and characterization of the postulated anaerobic ribonucleotide reductase.

Acknowledgements

This study was supported by Deutsche Forschungsgemeinschaft, Sonderforschungsbereiche 103 and 323, and by Fonds der Chemischen Industrie. We thank Prof. P. Reichard and Dr. T. Barlow for stimulating discussions.

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Nachdruck – auch auszugsweise – nur mit schriftlicher Genehmigung des Verlages gestattet
Verantwortlich für den Inhalt: A. KLEMM
Satz und Druck: Allgäuer Zeitungsverlag GmbH, Kempten