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Short Communications

Altered Ribonucleotide Reductase Obtained by *in vitro* Mutagenesis of Cloned *Escherichia coli* DNA *

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Ribonucleotide reductase[1] is the enzyme responsible for the conversion of ribonucleotides to deoxyribonucleotides and possesses a key role in the interplay of precursor to DNA synthesis. In *Escherichia coli* the enzyme consists of two subunits, proteins B1 (mol. wt. 160 000) and B2 (mol. wt. 78 000). The B1 subunit contains binding sites for allosteric effectors and all four ribonucleotide substrates. The B2 subunit contains two nonheme irons and a tyrosine free radical which is involved in the catalytic process. A 1:1 association of the two subunits forms the enzymatically active holoenzyme.

We decided to try to mutagenize the genes of B1 and B2 for the following reasons: (i) Mutations in the B1 protein might result in changes in the allosteric regulatory pattern, generating an unbalanced supply of deoxyribonucleotides for the replication machinery and might therefore affect DNA replication. (ii) Mutations in the B2 protein could resolve the structures needed to stabilize the tyrosine radical and its relation to the iron center. (iii) Mutations in either of the two subunits might also shed light on the possible association of ribonucleotide reductase with a higher order complex of proteins involved in precursor and DNA synthesis. Multicopy hybrid plasmids containing the two structural genes and a regulatory region for ribonucleotide reductase (*nrd*-region) had been constructed earlier.[2] Using such plasmids an efficient mutagenesis of the *nrd*-region is greatly favoured since this region comprises 20–30% of the total plasmid DNA.

Experimental. Covalently closed pPS 2[2] was nicked once at random with deoxyribonuclease I in the presence of ethidium bromide[3] and then gapped using the exonuclease function of DNA polymerase I. The gapped molecules were then treated with the single strand specific mutagene sodium bisulfite, and finally repaired by the polymerizing activity of polymerase I.[3] The mutagenized DNA (625 ng) was then transformed into *E. coli strain KK 535 (thr leu thi deo tonA lacY supE44 recA nalA nrdA nrdB)* according to the method given by Cohen *et al.*[4] Transformed cells were plated in soft agar on minimal agar plates supplemented with 0.4% glucose, 0.1% casamino acids, leucine (80 μg/ml), threonine (50 μg/ml), thymine (20 μg/ml), and tetracycline (10 μg/ml). KK 535 is sensitive to the drug hydroxyurea (>5 μg/ml) and also to incubation temperatures above 30 °C as a consequence of its *nrd*-lesions, whereas the same strain containing pPS 2 is resistant up to 2–3 mg/ml of hydroxyurea and still grows at 40 °C.[2]

The occurrence of mutations in the *nrd*-region was therefore screened by replica plating of transformants on hydroxyurea-containing plates or at high temperature. In all hydroxyurea-sensitive clones the presence of plasmid DNA of the proper size was verified using the method of Bernard and Helinski.[6] The amount of proteins B1 and B2 produced in the mutant strains were measured by rocket immunoelectrophoresis.[5] To measure enzymatic activity, a modified version of the disc assay system[7] was used. In order to obtain maximal reductase activity in plasmid containing strains, approximately 300 pmol of thioredoxin, 40 pmol of thioredoxin reductase and 1.2 nmol of NADPH were added to about 5×10^7 cells on each cellophane disc. This assay measures the enzymatic activity of the holoenzyme synthesized within the cells. The enzymatic activity of each subunit separately was measured in the presence of an excess of the other purified subunit (36 pmol of protein B1 or 28 pmol of protein B2).

Results and discussion. Of the 2000 obtained transformants, 23 had significant reduced resistance to hydroxyurea; two of them were also temperature sensitive for growth. Table 1 shows immunological and enzymatic measurements of proteins B1 and B2 in some of the isolated mutants, as well as the corresponding values for KK535 carrying pPS 2.

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Table 1. Amounts and enzymatic activity of proteins B1 and B2 in KK 535 cells containing mutagenized pPS 2 plasmids.

Plasmid	Protein content					Type of mutation
	μg/mg of total protein		u[a]/mg of total protein			
	B1	B2	Holo activity	B1 addition	B2 addition	
pPS 2	32	14	55	45	50	No
d80	88	3	4	2	61	Nonsense B2
t31	7	25	2	18	4	Nonsense B1
k68	63	28	2	59	14	Missense B1
n65	45	29	2	2	49	Missense B2

[a] Unit: nanomol of dCDP formed per min at 37 °C.

The mutagenization method applied in this experiment results in GC to AT transitions.[3] The exonuclease activity of polymerase I exposes under our conditions on the average five nucleotides as single strand DNA.[8] The mutagenization should therefore predominantly result in point mutations, leading to *e.g.* missense or with lower frequency to nonsense mutants. Base changes in regulatory regions of the plasmid should occur with very low frequency due to the small target size of such regions. Comparison of the mutant and wild type values for the immunological and enzymatic levels of proteins B1 and B2 enables in many cases a prediction of the type of mutation obtained. In Table 1 one representative of each type is shown. Twelve of fifteen characterized mutants seem to be of missence type (k68 and n65 in Table 1) and three are conceivable of nonsense type (d80 and t31 in Table 1). The procedure applied in this experiment represents a useful approach for the construction and isolation of missense and nonsense mutations in an essential structural gene.

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