Synthetic Multifunctional Proteins

Isolation of Covalently Linked Tryptophan Synthetase α-Subunit-lac-Repressor-β-Galactosidase Chimeras

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Summary. Several E. coli mutants were isolated which produce triple chimeras between one of the trp enzymes lac, repressor and β-galactosidase. The mutants were isolated as TonB- Lac+ derivatives of a phenotypically Lac- TrpR- strain carrying a lacI+Z+ fusion on a φ80dλlac phage. The phage is integrated into the chromosome in such a way that the lac and the trp genes are transcribed in the same direction. Of a total of 58 candidates 2 TrpA- and 3 Trp+ strains produce triple chimeras. The chimeras from the two TrpA- strains were further examined. They consist of tryptophan synthetase α-subunit, lac repressor and β-galactosidase. In crude extracts of these strains the tryptophan synthetase α-subunit part can be identified by its ability to aggregate with the β-subunit since some of the β-subunit activity can be precipitated with antiserum against β-galactosidase. Furthermore β-galactosidase precipitates with antiserum against tryptophan synthetase α-subunit. The lac repressor part is able to bind IPTG, but not lac operator DNA in vitro. The β-galactosidase part is as unaffected as in the original lac repressor-β-galactosidase chimera. The molecular weights of both chimeras are 175,000 when determined by SDS gel electrophoresis. The chimeras are partially degraded giving rise to fragments of distinct molecular weights.

Introduction

Multifunctional enzymes, i.e. enzymes which carry two or more catalytic activities in one polypeptide chain (for review see Kirschner and Bisswanger, 1976) offer a way to study the evolution of complex proteins. They most probably evolved by fusion of two or more genes. One might consider two possible mechanisms for the fusing process both of which have been verified by isolation of “artificial” multifunctional proteins.

In the first case the fusion is the result of frame shift mutations. In such a way the hisD and hisC genes in Salmonella typhimurium have been fused, now producing a protein having the activities of both gene products (Yrormo et al., 1970).

In the second case a deletion taking out the translational start and stop signals fuses the two genes which now code for one polypeptide chain. Examples for this are the lac-repressor-β-galactosidase fusions in Escherichia coli (Müller-Hill and Kania, 1974). These chimeras were isolated as revertants of the highly polar ochre mutation Z Ul18 which maps at the operator proximal region of the Z-β-galactosidase-gene. The deletions begin within the lac I-gene and go beyond the ochre mutation; transcription of the Z-gene now starts at the I-gene promoter which carries the F mutation (Müller-Hill et al., 1968). Recently an elegant method has been described to enable the fusion of β-galactosidase to nearly every protein in E. coli (Casadaban, 1976). Thus chimeras between β-galactosidase and the araC gene product (Casadaban, 1976), and between β-galactosidase and the malF gene product have been generated (Silhavy et al., 1976).

In this work we describe triple chimeras, consisting of the tryptophan synthetase α-subunit, lac repressor and β-galactosidase. We isolated the strains producing these “monsters” by using the classical trp-lac operon fusion system first described by Beckwith et al. (1966). We started with a strain already containing a lac-repressor-β-galactosidase chimera. We did these experiments to test whether there exists an upper limit for protein fusion in respect of molecular weight and number of components.
Materials and Methods

Abbreviations. IPTG, isopropyl-β-D-galactosidase; SDS, sodium dodecyl sulfate; Tris, Tris-hydroxy-methyl-amino-methan; X-gal, 5-bromo-4-chloro-3-indoly-β-D-galactosidase.

Media and Plates. (Contents per 1000 ml of ion exchanged water) DYT-broth: Yeast (Difco) 10 g, Tryptone (Difco) 16 g, NaCl 5 g. Minimal plates: Na₂HPO₄·x2H₂O 7 g, KH₂PO₄ 3 g, NH₄Cl 1 g, Na₂SO₄ 0.8 g, MgSO₄ 0.3 g, Na citrate 1 g, thiamine 50 mg, sugar (carbon source) 4 g, agar (Difco) 15 g. For ara fuc plates 1 g of L-arabinose and 2 g of D-fucose were used. If needed: amino acids 100 mg, streptomycin 200 mg, x-gal (Cyclo) 50 mg, 5-methyl-DL-tryptophan (Sigma) 50 mg. Other plates used and most of the bacterial and phage techniques are as described by Miller (1972).

Strains. The starting strain for the selection of protein fusions between one of the trp enzymes and the lac-repressor-β-galactosidase fusion was GH 51-72-1 (lac-pro)₃ thi strA trpR φ80dlac I⁻-Zφ80vir 51-76-14.

The strain was constructed as follows: X7700 (lac-pro)₃ thi strA araφ80dlac = was mated with D 7011 (HfrC thi trpR). Ata⁻ Str₃ recombinants were selected and tested for resistance against 5-methyl-DL-tryptophan. Into this strain (GH 51-1 (lac-pro)₃ thi strA trpR φ80dlac =) the epism of BMH 72-16-2 (lac-pro)₃ thi F⁻ lac pro (lac-pro)₃ thi strA trpR 4)80dlac was crossed. Pro₃ Str₃ derivatives were selected and homogenates with Lac⁻ phenotype were isolated from EMB-lac plates. Then the epism was cured with acridine orange. WD 5017 thiF colV, B⁺ trp⁺ cyrR⁺ was used for the preparation of colicins V and B. We used BMH Hfr H 335-1 (lacwa) thi araC⁰ to get trp R⁻ derivatives of the proposed fusion strains. The φ80vir phages and the strains X7700, D7011 and WD5017 were taken from the Cold Spring Harbor collection.

Selection Procedure for Mutations in the tonB Locus. Lysates of φ80vir phages and colicins V and B were prepared as described by Gottesman (1969). Phage lysates with a titer of >10⁵ were and colicin preparations with a killing factor of >5 x 10⁸ were used. 2.5 ml of an overnight culture were spun down and the cells resuspended in 0.2 ml φ80vir lysate and 0.2 ml of the colicin lysate. The mixture was incubated for 20 min at 37°C, then 0.2 ml was plated onto a McConkey lac plate, which was incubated for 2 days at 37°C. In average 300 TonB⁺ colonies grew per plate.

TMS II Buffer. 0.2 M NaCl, 10⁻⁴ M EDTA, 0.01 M Mg acetate, 0.01 M Tris pH 7.5, 0.05 M Mercaptoethanol (Gilbert and Müller-Hill, 1966).

β-galactosidase Assay. The assay was carried out as described by Miller (1972) with the exception that full medium was used. 1 ml of each culture was centrifuged, the cells resuspended in 1 ml of assay buffer and disrupted by sonication for 30 s with a Branson sonifier.

IPTG Binding Test. The method of Gilbert and Müller-Hill (1966) was used.

Lac Operator Binding Assay. Binding of lac operator DNA was assayed as described by Riggs et al. (1970). As crude extracts were used for the test, it was carried out in the presence of a hundred fold excess of chicken blood DNA over ³²P-labeled DNA to chase unspecific DNA binding. No bovine serum albumine and no dimethyl sulfoxide was added to the binding buffer.

Assay for the β-Subunit of Tryptophan Synthetase. The conditions for the test were the same as described by Smith and Yanofsky (1963) with the exception that ¹⁴C-indole was used as the substrate. The non-metabolized indole was extracted after the reaction with 4 ml of toluene. 1 ml of the organic phase was counted in 10 ml of Bray's solution in a liquid scintillation counter.

SDS Gel Electrophoresis. The method described by Laemmli (1970) was used. To guarantee complete reduction of the antibody precipitates the reduction buffer contained 5% of SDS and 5% of BME. The samples were kept for 10 min in a boiling waterbath for reduction and denaturation.

Preparation of Antisera. Two randomly bred rabbits were immunized with E. coli protein. 1 mg of the respective protein was dissolved in 1 ml of 0.15 M NaCl and 1 ml of Complete Freund's Adjuvant (Difco). The whole suspension was used to immunize one rabbit. After 4 weeks the procedure was repeated with the exception that Incomplete Freund's Adjuvant was used. Ten days after the second immunization the rabbits were bled by heart puncture. The blood was allowed to coagulate overnight in the cold, the serum was taken with a pasteur pipette.

Results

Selection of Fusion Strains

The genetic system in which deletions can be selected which fuse the lac operon to the trp operon has previously been described by Beckwith et al. (1966). The lac operon in parent strains for such fusions is translocated by a φ80dlac transducing phage to the att₈₀ site on the chromosome near the trp operon. Between the trp and the lac operon lies the tonB locus, which determines sensitivity to T1 and φ80 phages and to Colicin V and B. The φ80dlac is integrated into the chromosome in such a way that the trp and the lac operon are transcribed in the same direction. Selection of spontaneous TonB⁻ derivatives in such strains often results in deletions which fuse the lac operon to the trp operon. The strain we used to isolate derivatives with fused gene products is shown in Figure 1 (For construction see Materials and Methods). Promoter and operator of the lac operon are deleted.

Fig. 1. Map of the trp-lac region of the starting strain and classification of the obtained operon fusions.