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Construction and characterization of a recombinant tripartite enzyme, galactose dehydrogenase/β-galactosidase/galactokinase

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The in-frame gene fusion between 3 enzymes, galactose dehydrogenase, β-galactosidase and galactokinase, is described. The purified artificial tripartite enzyme displayed all three enzymic activities. Two major forms of the hybrid protein were found, consisting of 4 and 8 subunits respectively, but other forms could also be identified. Each subunit was made up of one monomer each of galactose dehydrogenase, β-galactosidase and galactokinase. Proximity effects exhibited by the hybrid enzyme could be demonstrated using [^{14}C]galactose as a reporter molecule.

Gene fusion; Galactose dehydrogenase; β-galactosidase; Galactokinase

1. INTRODUCTION

Substantial benefits of an artificial bifunctional enzyme relative to the corresponding native enzymes have been obtained by the fusion of two genes, coding for two sequentially operating enzymes (for reviews on naturally occurring multifunctional enzymes see [1,2]). Distinct differences in transient time and steady state rate of the coupled reaction were observed when an earlier studied hybrid enzyme, β-galactosidase/galactose dehydrogenase, was compared with an identical system composed of native enzymes [3]. In addition, with another system, β-galactosidase and galactokinase fused in-frame to form a bifunctional enzyme, the intermediate product, galactose, was more efficiently transferred to the second enzyme in the hybrid than to a competing enzyme present, galactose dehydrogenase [4,5]. To further elucidate such hybrid enzymes a trifunctional enzyme, galactose dehydrogenase (*Pseudomonas fluorescens*/β-galactosidase (*E. coli*) was prepared by gene fusion. These enzymes catalyze the sequential hydrolysis of lactose followed by either the oxidation of the galactose formed to the corresponding lactone or the phosphorylation of galactose to galactose-1-phosphate (Fig. 1). In addition to providing fundamental information about proximity effects, stability and subunit aggregation, the described hybrid protein should find an important field of application in carbohydrate analyses.

2. MATERIALS AND METHODS

2.1. *Chemicals and reagents*

The enzymes used for DNA manipulation, β-galactosidase and galactose dehydrogenase were purchased from Boehringer Mannheim; [1-^{14}C]galactose was from Amersham. All other reagents were commercially available and of analytical grade.

2.2. *Bacterial strains, plasmids and indicator plates*

E. coli strain F'11 *recA* ((lac,pro) Δthi, rifA, strA, recA/F'lacI^{q}Z$^-$, pro$^+$) [3] and *E. coli* strain C 600K$^-$ (galE$^-$ T^r K^m, lac$^-$, thr$^-$, leu$^-$) [4] were used for standard transformation procedures. The plasmids pZK 205 and pDZ 10 have been described earlier [3,6]. *LacZ*$^+$ colonies were identified on indicator plates containing Xgal and ampicillin. Galactokinase positive transformants were detected as red bacterial colonies on McConkey galactose plates [4].

2.3. *Construction of plasmids*

Restriction enzyme digests and other cloning procedures were performed as described by Maniatis et al. [7].

2.4. *Purification of the tripartite protein*

The fusion protein was purified according to the procedure developed for the corresponding bifunctional enzymes [3,8].

2.5. *Enzyme assays*

During purification, β-galactosidase was assayed by hydrolysis of 0.8 g/l ONPG [3] in a buffer consisting of 0.1 M NaH_2PO_4, pH 7.0 and 1 mM $MgSO_4$. One unit of enzyme hydrolyzes 1 μmol of lactose per min at room temperature. This corresponds to the hydrolysis of 17 μmol ONPG per min. Galactose dehydrogenase activity was determined with 16.6 mM galactose as substrate [9]. One unit of galactose dehydrogenase oxidizes 1 μmol of galactose per min at room temperature in a buffer consisting of 90 mM Tris-HCl, pH 8.5 and 0.5 mM NAD. Galactokinase activity was determined using [^{14}C]galactose [10]. One unit of galactokinase phosphorylates 1 μmol of galactose per min at room temperature in 0.1 M Tris-HCl pH 8.0 containing 4 mM $MgCl_2$, 1.6 mM ATP, 3.2 mM NaF and 1 mM DTT.

2.6. *Determination of pH profiles*

Enzyme activities were determined in 0.1 M Tris-HCl containing 59 mM $MgCl_2$ in the pH range from 7.0 to 10.0. In the case of galactokinase, 3.2 mM NaF and 1 mM DTT were added to the buffer.

2.7. *Proximity effects*

A competitive assay system was set up to monitor the transfer of the galactose formed by the β-galactosidase moiety of the tripartite enzyme to be converted to either galactono-lactone or galactose-1-phosphate and was used to measure differences between

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Fig. 1. Reactions catalyzed by the artificial trifunctional enzyme galactose dehydrogenase/β-galactosidase/galactokinase.

the tripartite enzyme and native enzyme systems. Buffer B (20 mM lactose and 0.5 mM NAD in 0.1 M Tris-HCl pH 8.0 containing 59 mM $MgCl_2$) was used in this assay and the rate of NADH formation was followed spectrophotometrically in the absence and presence of 1.6 mM ATP.

In order to ensure that the activities of the native and tripartite enzymes were matched, the galactose dehydrogenase activity was monitored using 16.6 mM galactose and 0.5 mM NAD. The β-galactosidase activity was monitored in the same buffer containing 38 mM lactose, 0.5 mM NAD and an excess of galactose dehydrogenase. Finally, the galactokinase activity was matched using labelled galactose. 1 ml of tripartite enzyme solution corresponds to 0.2 mU β-galactosidase, 2.4 mU galactose dehydrogenase and 0.08 mU galactokinase at pH 8.0. The appropriate amount of the hybrid enzyme (10 μl) or native enzymes with separate activities equal to those of the fusion protein was added to the assay solution. In order to evaluate any proximity effects of the galactokinase moiety 0.1 mM labelled galactose was included in the assay mixture and the amount of galactose-1-phosphate formed was determined in a total volume of 100 μl.

3. RESULTS AND DISCUSSION

3.1. *Construction of pDZK 1*

A schematic representation of the plasmid pDZK 1, encoding an in-frame fusion between the structural genes of galactose dehydrogenase, β-galactosidase and galactokinase, is outlined in Fig. 2. pZK 205, which encodes β-galactosidase/galactokinase, was initially digested with *Sac*I and *Sca*I. The 3.8 kb DNA fragment

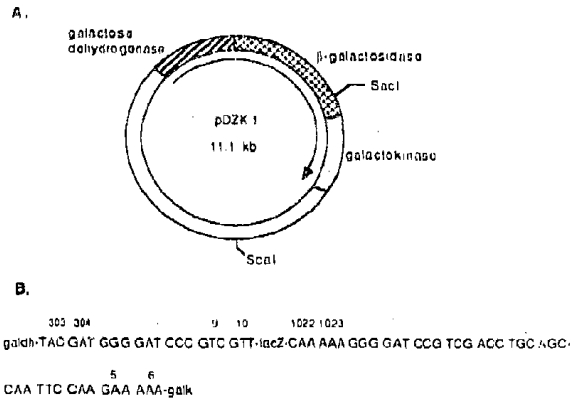

Fig. 2. (A) Schematic representation of the chimeric plasmid pDZK1 coding for an in-frame fusion between galactose dehydrogenase, β-galactosidase and galactokinase. (B) Nucleotide sequence of the linker regions between the fused galactose dehydrogenase (*galdh*), β-galactosidase (*lacZ*) and galactokinase (*galk*) genes. The numbers indicate the amino acid residue numbers of the galactose dehydrogenase, β-galactosidase and galactokinase, respectively.

encoding the carboxylic part of the *lacZ/galK* gene was inserted into pDZ 10 digested with the same enzymes. Use of *E. coli* C600K⁻ as host cell resulted in *galk*⁺ transformants. When transformed into *E. coli* F' 11 *recA*, selection on indicator plates yielded *lacZ*⁺ transformant colonies which were screened for plasmids with a size of 11.1 kb. One of the isolated plasmids, pDZK 1, encodes a polypeptide of 1711 amino acid residues carrying the galactose dehydrogenase, β-galactosidase, and galactokinase activities.

3.2. *Purification of galactose dehydrogenase/β-galactosidase/galactokinase*

β-galactosidase adsorbs strongly to DEAE-Sepharose, which makes purification of the hybrid very simple. The elution profiles from ion exchange chromatography on DEAE-Sepharose and gel filtration on Sephacryl S-400 Superfine showed that the 3 enzyme activities eluted together. The specific activities of the tripartite enzyme were 5.1 U/mg for galactose dehydrogenase, 2.1 U/mg for β-galactosidase and 0.11 U/mg for galactokinase. When corrected for the increase of M_r caused by the gene fusion, these specific activities correspond to 20–30% of native galactose dehydrogenase and β-galactosidase and 70–80% of native galactokinase. The molecular mass of the fusion protein was determined by gel filtration and SDS-polyacrylamide gel electrophoresis. The protein purified by gel filtration eluted as two main peaks (Fig. 3), corresponding to M_r values of 1500 and 750 kDa; peaks corresponding to larger aggregates were also observed. As estimated from the SDS-PAGE, the subunit mass of the hybrid protein is 190 000. These data suggest that the fusion protein is present in two principal forms, a tetrameric and an octameric form. The hybrid β-galactosidase/galactose dehydrogenase has been shown to exist mainly as hexamers and tetramers, with a preference for the hexameric form [3]. The galactokinase part of the tripartite enzyme thus modifies the form of protein aggregation.

3.3. *Thermostability*

The β-galactosidase and galactokinase moieties of the hybrid proved to be more sensitive to heat denaturation than the native enzymes (Fig. 4). In contrast, native galactose dehydrogenase was less heat-stable than the galactose dehydrogenase part of the tripartite enzyme. The same phenomena have been observed with β-galactosidase/galactokinase and β-galactosidase/galactose dehydrogenase [3].

3.4. *Determination of pH profiles*

In the pH range tested, the maximal activity of the β-galactosidase and galactokinase parts of the fusion enzyme was found at pH 7.0 and 9.0, respectively, the same as for the native enzymes. The galactose

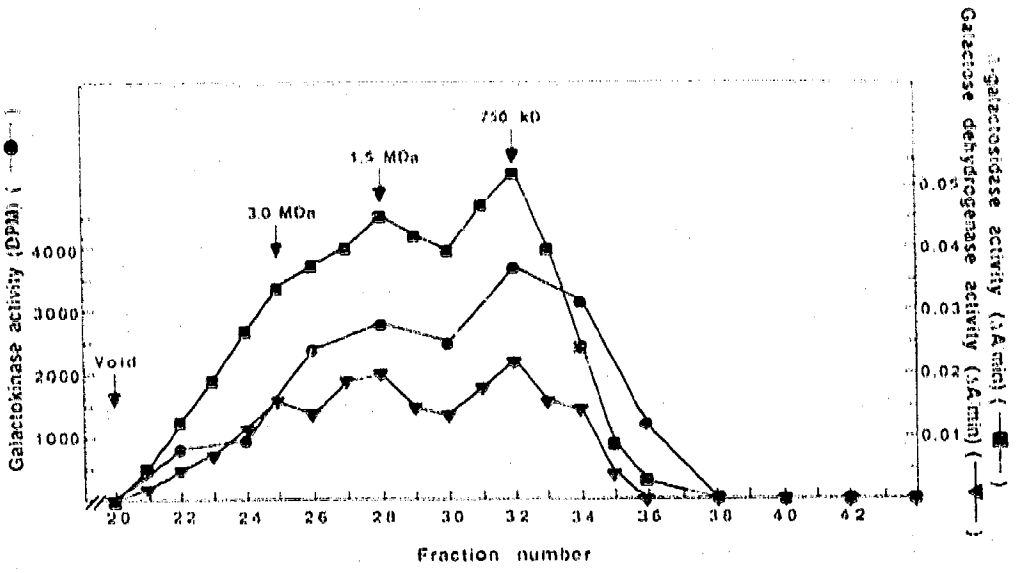

Fig. 3. Gel filtration chromatography on Sephacryl S-400 Superfine of the tripartite enzyme. Fraction volume: 3 ml.

dehydrogenase moiety exhibits a slight shift in pH optimum, with maximal activity at pH 9.0, while native galactose dehydrogenase showed a pH optimum of 10.0.

3.5. *Proximity effects*

In order to investigate the proximity effects and potential channeling of galactose to either the galactokinase or the galactose dehydrogenase moiety of the hybrid protein, a spectrophotometric assay was utilized. The rate of NADH formation was monitored in the presence and absence of ATP. The reaction rate proved to be completely unaffected by the addition of ATP, indicating that the galactose formed was efficiently transferred to the dehydrogenase moiety. This is in agreement with our previously obtained data from corresponding bifunctional enzymes, since such proximity effects are more pronounced in β-galactosidase/galactose dehydrogenase than in β-galactosidase/galactokinase. This is partly due to the higher intrinsic activity of the dehydrogenase than the kinase, but other factors such as a more favourably oriented active site or a more efficient path of galactose diffusion in the former case must also be involved. However, the galactokinase part of the hybrid enzyme also exhibits proximity effects in the absence of NAD. This was demonstrated with [^{14}C]galactose as a reporter molecule. Thus, with ATP, lactose and [^{14}C]galactose in the assay solution a competitition occurs between the labelled galactose and the galactose produced by hydrolysis of lactose. In the hybrid protein the galactose formed appears to be channeled to the galactokinase moiety since the amount of produced [^{14}C]galactose-1-phosphate only reached 0.067 nmol/min while the value obtained without lactose present was 0.17 nmol/min. However, when native enzymes with the same activity as the tripartite enzyme were used, the corresponding value, with lactose present, was 0.13 nmol/min. Such channeling or proximity effects can be most valuable when these enzymes are utilized for biochemical analyses of lactose and galactose [11,12]. Furthermore galactose dehydrogenase also accepts other substrates besides galactose including arabinose, which thus interferes with lactose and galactose determinations. Galactokinase is, however, specific for galactose and this enzyme moiety can be used separately to phosphorylate the galactose present, thereby avoiding oxidation by galactose dehydrogenase. When analyzing complex carbohydrate mixtures it is therefore important to be able to regulate the individual enzymatic activities of the hybrid enzymes.

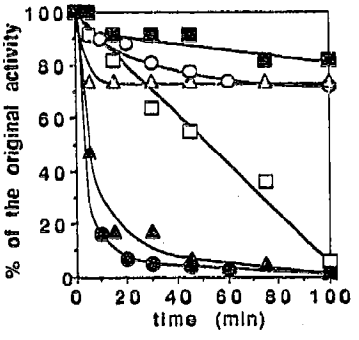

Fig. 4. Heat stability measurements of the tripartite and the native enzymes were carried out at 50°C for the indicated length of time in 0.1 M Tris-HCl pH 8.0 containing 1 mM $MgCl_2$ and 1 mM DTT, and residual activities were determined. Bovine serum albumin was added to give a final protein concentration of 1 mg/ml. (-▲-) Native β-galactosidase; (-▲-) hybrid β-galactosidase; (-□-) native galactose dehydrogenase; (-■-) hybrid galactose dehydrogenase; (-○-) native galactokinase; (-●-) hybrid galactokinase.

This can be achieved by adjusting cofactor and metal ion composition of the buffer [12].

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