DETECTION BY AFFINITY METHODS OF A PLASMID-CODED ENZYME (GUANINE PHOSPHORIBOSYLTRANSFERASE) IN A COUPLED TRANSCRIPTION/TRANSLATION SYSTEM FROM *ESCHERICHIA COLI*

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1. Introduction

The cell-free coupled transcription/translation system from *E. coli* originally developed by Zubay [1,2] and afterwards modified by many authors is one of the best tools for detecting plasmid-coded proteins. Because the system is also able to transcribe and translate information from small DNA fragments (see [3]) it is of great importance for proving the integrity of genes in the course of cloning experiments.

Until now the following methods have been used for detection of specific gene products:

(i) Identification of individual bands in electrophoretic patterns (see [4]);
(ii) Immunoprecipitation with monospecific antibodies [5–7];
(iii) Assay for a specific function like enzyme activity [1,6,8] or toxicity in the case of bacteriocins [9,10].

Method (i) gives no information about the functional activity of the protein. For method (ii), one needs antibodies against the highly purified enzyme and for using method (iii), the system must be prepared from an *E. coli* strain defective in the respective function.

Because of these limitations we developed two new specific detection methods based on the affinity of an enzyme (guanine phosphoribosyltransferase) to its substrate or reaction product (guanosine monophosphate) coupled on a matrix. The methods will be described and compared with those mentioned above concerning the expression of the *gpt* gene on a ColE1-derived plasmid.

2. Materials and methods

2.1. *Chemicals*

GMP, XMP and phosphoribosylpyrophosphate were from PL-Biochemicals (USA), all 4 triphosphates from Calbiochem, amino acids from Reanal (Hungary), Sepharose 4B and Sephadex G-25 from Pharmacia, DE-81 filter discs from Whatman, DNase from Miles, and micrococcal nuclease from Boehringer.

2.2. *Bacteria*

Escherichia coli Q13 (RNase-, polynucleotidphosphorylase-) was obtained from Dr Inselburg [11], *E. coli HfrH gpt hpt* from Dr Livshitz [12], and *E. coli* JA 200 containing the plasmid pLC 44-11 from Dr Carbon [13].

2.3. *Preparation of S30*

The standard S30 was prepared from *E. coli* Q13 ($RNase^-$, DNase I^-) as in [14] and outlined here only in short. Frozen cells (3 g) were homogenised by grinding with 3 g glass beads (0.18–0.20 mm diam.) and 4.5 ml buffer A [1] N_2 frozen. After centrifugation at 30 000 × *g* the supernatant was incubated for 1 h at 37°C with DNase–Sepharose [15] for degradation of endogenous DNA. Endogenous mRNA was destroyed by subsequent incubation with 10 μg/ml micrococcal nuclease [16,17] in the presence of 1 mM $CaCl_2$ for 15 min at 20°C. The nuclease reaction was stopped by addition of 2 mM EGTA and the extract was filtered through Sephadex G-25 in buffer A. The eluate was frozen in small aliquots in N_2.

An S30 from the defective strain *E. coli HfrH gpt hpt* was prepared in a similar way.

2.4. *Preparation of affinity matrices*

GMP–Sepharose was prepared as in [18]. The same method was also applied for XMP–Sepharose coupling. The maximal binding in both cases was ~1 μmol/ml gel.

Binding of GMP and XMP to DEAE filter-disks was achieved by incubation of the filters for 2 h with 5 mM solutions of the respective monophosphate. After extensive washing with water the filters were incubated for 15 min with 1% amino acids at pH 7, washed and dried.

2.5. *Use of the affinity matrices*

For removal of the guanine phosphoribosyltransferase from the Q13 system the extract was applied onto a 0.3 ml column of GMP– or XMP–Sepharose after the nuclease digestion and was eluted with buffer A.

The cell-free synthesised ^{3}H-labeled enzyme was precipitated from the incubation mixture by addition of 10 μl GMP– or XMP–Sepharose, centrifuged, washed with buffer A and counted for radioactivity. Alternatively the cell-free incubation mixtures were applied onto the ligand–DEAE filter-disks. The filters were immediately put into a 1% amino acid solution (pH 7) and after 15 min washed with several changes of water. For elution of the guanine phosphoribosyltransferase they were immersed for 15 min in 1 mM GMP.

2.6. *Transcription/translation assay*

Protein synthesis was performed with the incubation mixture of [2] in 30 μl total vol. for 1 h at 37°C. Each tube contained 1.5 μCi [^{3}H]leucine. Total protein synthesis was measured after application of 10 μl mixture to filter paper discs impregnated with 5% trichloroacetic acid (TCA), washing the filters with cold 5% TCA in acetone, heating in 5% TCA and washing with ether/ethanol [14].

2.7. *Immunoprecipitation*

Of a 10% suspension of *Staphylococcus* (Cowen I strain) in 25 mM Tris–NCl (pH 7.4)–10 mM EDTA–0.35 M NaCl–0.15% Triton (TENT) [19] 20 μl were added to the cell-free incubation mixture. Unspecific precipitates were centrifuged off and 10 μl solution of antibodies against the purified guanine phosphoribosyltransferase (M.S., unpublished) were added to the supernatant. After incubation overnight at 4°C 20 μl of the *S. aureus* suspension were added. After incubation for 15 min at 0°C the precipitate was centrifuged and washed 4 times with buffer TENT. The immunoprecipitate was usually denatured in SDS–buffer for electrophoresis and an aliquot (5 μl) was taken for counting of radioactivity.

2.8. *Enzyme assay*

The activity of the guanine phosphoribosyltransferase was assayed as in [20] for the eukaryotic enzyme with 0.14 mM (60 nCi) [^{14}C]guanine and [^{14}C]xanthine.

Of the protein synthesis mixture, 20 μl, stopped by 25 μg/ml chloramphenicol and allowed to stand overnight for association of enzyme subunits, were incubated in the enzyme assay.

2.9. *Plasmid isolation*

The plasmids pLC 44-11 and Col E1 were isolated by lysozyme–EDTA–detergent treatment of the bacteria and purified by CsCl gradient centrifugation as in [21].

3. Results

The S30 system from *E. coli* Q13 contains normal guanine phosphoribosyltransferase and, therefore, can not be used for the cell-free synthesis of this enzyme. An S30 from the enzyme-deficient strain *E. coli HfrH gpt hpt* has a residual enzyme activity of almost 1%. To obtain a system without any detectable endogenous enzyme activity, we tried to remove the guanine phosphoribosyltransferase by binding to GMP– or XMP–Sepharose. As can be seen from table 1, no enzyme activity was detectable after different treatments. But the treatment with GMP–Sepharose in buffer A results in a total loss of protein synthesising capacity because of binding of translation factors. This effect can partially be avoided by elution from the GMP–Sepharose with 1.2 M KCl. The best system with relatively high protein synthesis rate results from XMP–Sepharose treatment in the presence of 1.2 M KCl which seems to be highly specific for guanine phosphoribosyltransferase.

Whereas the endogenous enzyme activity of the mutant system was exceeded only up to 3-fold upon DNA-directed protein synthesis with the *gpt* gene con-

Table 1
Total protein synthesis and relative activities of guanine phosphoribosyl-transferase with differentially pretreated systems

Pretreatment	cpm Precipitable by trichloroacetic acid	% Enzyme activity
Standard Q13	142 520	100
GMP–Sepharose	660	0.1
GMP–Sepharose + 1.2 M KCl	62 300	0.1
XMP–Sepharose + 1.2 M KCl	102 230	0.1
Standard *E. coli* *HfrH gpt hpt*	78 300	1

Aliquots (10 μl) of incubation mixtures were used for precipitation but the cpm are given for 30 μl. Protein synthesis was done with 4 μg pLC 44-11. The enzyme activities are determined only from the S30 in this case

Table 2
Enzyme activities of guanine phosphoribosyltransferase after incubation of different DNAs in the S30 of the mutant strain and in the XMP–Sepharose treated system, respectively

S30	DNA	cpm Guanine converted
Standard *HfrH gpt hpt*	–	720
	Col E 1	640
	pLC 44-11	2150
Q13/XMP –Sepharose	–	120
	Col E 1	90
	pLC 44-11	1700

Col E1 DNA (1 μg) and pLC 44-11 DNA (4 μg) were incubated in the system. Enzyme activities were determined 20 h later by incubation for 1 h. From each cpm value the unspecific substrate binding of 320 cpm was subtracted

taining plasmid pLC 44-11 [22], this plasmid stimulates enzyme synthesis in the XMP–Sepharose-treated Q13 system which results in an enzyme activity 14-times higher than the background (table 2). With neither method can the enzyme activity be detected after cell-free protein synthesis directed by the ColE1 plasmid from which the hybrid plasmid pLC 44-11 is derived.

The synthesis of guanine phosphoribosyltransferase in the coupled system can also be detected by immunoprecipitation of radioactively labeled protein with specific antibodies (table 3). Nearly the same amount of labeled protein is precipitable with the GMP–Sepharose matrix. The slightly higher value for the immunoprecipitate seems to result from small amounts of unspecific precipitates as detected by electrophoresis (not shown). The labeled protein from both precipitates migrates to the same position as the purified enzyme in SDS electrophoresis (M. S., in preparation). We further tested the usefulness of DEAE filter-discs with prebound GMP as an affinity matrix for specific binding of the guanine phosphoribosyltransferase. As can be seen from table 4 the value of bound radioactivity is higher in the case of incubation with pLC 44-11 than with unspecific pro-

Table 4
Specific binding of guanine phosphoribosyltransferase to GMP–DEAE filter-disks

DNA	cpm After washing	cpm After elution with GMP	Difference
–	1150	1115	35
Col E 1	1250	1190	60
pLC 44-11	1870	1120	750

10 000 cpm were applied onto the filters in each case

Table 3
Comparison of immunoprecipitation and affinity precipitation of labeled guanine phosphoribosyltransferase synthesized in the S30 of Q13

DNA	cpm Total protein	Immunoprecipitate	GMP–Sepharose bound
–	5560	380	260
Col E 1 (1 μg)	88 600	435	240
pLC 44-11 (4 μg)	147 200	8315	6755

From each 30 μl incubation mixture 10 μl were precipitated with TCA, 10 μl were subjected to immunoprecipitation, and 10 μl were treated with an equal volume of GMP–Sepharose. All values are given for 30 μl

tein synthesis. After elution with 1 mM GMP no difference can be detected. Therefore, the eluted radioactivity belongs to the guanine phosphoribosyltransferase as was also confirmed by electrophoresis (not shown).

4. Discussion

We have demonstrated the cell free synthesis of guanine phosphoribosyltransferase directed by the *gpt* gene containing plasmid pLC 44-11 [22]. But the enzyme activity value obtained after synthesis in a mutant S30 system was only 3-times higher than the residual endogenous activity of the system. Now we have developed two new types of detection methods:

(1) The endogenous enzyme is removed by binding to the specific affinity column allowing the detection of enzyme activity with high sensitivity.
(2) The radioactively labeled newly synthesised enzyme is precipitated by substrate–Sepharose or is specifically bound to substrate filters.

The filter binding method is the most rapid but has the disadvantage of a relatively high degree of unspecific binding. The substrate–Sepharose precipitation is comparable with immunoprecipitation but is more rapid, specific for the enzyme like pure monospecific antibodies and does not require purification of the enzyme and antibody production. Removal of the endogenous enzyme from the S30 is, in our opinion, the best method to determine cell-free synthesis of active enzymes but is applicable only if the matrix bound substrate does not bind any component of the transcription/translation system, like GMP in our case.

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