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ANALYSIS OF THE PROTEIN-KINASE ACTIVITY OF *ESCHERICHIA COLI* CELLS

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SUMMARY *When growing E. coli cells in a minimal medium containing radioactive orthophosphate, several proteins appear to be significantly labeled after analysis by polyacrylamide gel electrophoresis and autoradiography : at least four in the soluble cellular fraction, five in crude ribosomes and one in salt-washed ribosomes. In all cases, phosphorylation occurs at the level of threonine and serine residues as shown by paper electrophoresis of acid hydrolysates of these proteins. It is concluded that bacteria do contain a protein kinase activity.*

INTRODUCTION

Several attempts have been made to demonstrate the presence in procaryotes of protein kinases, the enzymes that catalyze the transfer of phosphoryl groups from adenosine-triphosphate or guanosine-triphosphate to serine or threonine residues of proteins. On the basis of *in vitro* as well as *in vivo* experiments, it has been reported that such enzymes would exist namely in *Escherichia coli* (1, 2, 3), in various strains of *Streptococcus* (4) and in *Salmonella typhimurium* (5). However, except in the latter instance, no definite evidence for phosphorylated proteins has been obtained so far.

In the particular case of *E. coli*, the originally described phosphorylating activity was found to be questionable (6) and it is now believed to be attributable to a polyphosphate kinase rather than to a protein kinase (7). Only in the special situation of bacteriophage T_7 infection a protein kinase activity

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has been demonstrated in bacteria by showing mainly its ability to phosphorylate the serine residues of endogenous proteins (8, 9). But, in fact, this enzyme appears to be naturally absent in host cells since it is coded for by a specific phage gene which has been mapped (10). In view of these data, it has been then concluded that there is no protein kinase activity in uninfected bacteria (10, 11).

In the present work, this problem was re-investigated by measuring the degree of *in vivo* orthophosphate incorporation into soluble and ribosomal proteins of *E. coli* and analyzing the nature of chemically modified products. The results presented hereafter indicate that a protein kinase activity does exist in this bacterial species.

MATERIALS AND METHODS

The previously described (12) *E. coli* strain CP 78 (arg^-, leu^-, thr^-, his^-, thi^-, rel A^+) was used through all experiments. Cells were grown for 2-3 generations in exponential phase at 37°C in a low-phosphate medium at pH 7.5 containing per liter : 12 g Tris, 2 g NH_4Cl, 2 g KCl, 0.5 g $MgCl_2$-$6H_2O$, 20 mg Na_2SO_4, 1.25 g casein hydrolysate, 25 mg K_2HPO_4, 3 g glycerol, 5 mg thiamine, 2 mg $FeCl_3$ and 50 mCi carrier-free [^{32}P] orthophosphate (French C.E.A., Saclay). When the absorbance of the culture at 600 nm reached 0.7, cells were poured over crushed ice and collected by centrifugation for 10 min at 17 000 x g.

For preparing proteins, bacteria were first disrupted by alumina grinding (13) and the lysate was centrifuged for 20 min at 30 000 x g. The supernatant thus obtained was then centrifuged for 180 min at 225 000 x g in order to separate the soluble fraction (S_{225}) from the pellet of "crude" ribosomes. Soluble proteins were precipitated with 10 % trichloroacetic acid (TCA), re-dissolved in 66 % acetic acid in 0.01 M Tris-HCl buffer at pH 7.7/0.07Mmagnesium acetate, dialyzed overnight against 6 % acetic acid and finally lyophilized. They were then solubilized (1 mg/ml) in a medium containing per liter : 8.4 g bistris-acetic acid (Sigma Chem. Co.) buffer at pH 6.0, 10 g sodium dodecylsulfate (SDS), 10 ml β-mercaptoethanol and 480 g urea. In some experiments, proteins were dissolved in the same medium except that the pH was brought to 6.8 and urea was omitted, and were incubated in presence of either pancreatic ribonuclease (1 mg/ml) for 6 h at 30°C or pronase (1 mg/ml) for 3 h at 30°C. After this, they were lyophilized then solubilized in the same medium as above at pH 6.0 in presence of urea. In other experiments, proteins were incubated for 36 h at 30°C with alkaline phosphatase as reported (14). Ribosomal proteins were prepared

from either "crude" ribosomes or "washed" ribosomes obtained after overnight incubation with 1 M NH_4Cl (15). Extraction was carried out following the acetic acid procedure (16), and proteins were dialyzed against 1 M acetic acid and finally lyophilized. When required, ribonuclease or pronase or phosphatase treatment was performed under the same conditions as those described above.

Soluble and ribosomal proteins (200-300 µg) were subjected to one-dimensional electrophoresis in 10 % polyacrylamide gels containing 1 % SDS (17, 18). Migration was carried out at 20°C in 0.2 cm-thick and 10 cm-long gel slabs under 150 volts. Reference proteins (about 1 µg each) of known molecular weights were analyzed simultaneously. After electrophoresis, gels were systematically incubated in 16 % TCA for 45 min at 90°C then in 5 % TCA for 12 h at room temperature (19). Proteins were stained with Coomassie blue as already described (20). Gels were then incubated for 1 h in a mixture containing 60 % methanol, 7.5 % acetic acid and 1 % glycerol, dried under vacuum and finally autoradiographed for 2-4 days (Kodirex films, Kodak).

The nature of phosphorylated amino acids was determined after acid hydrolysis (21) of total proteins prepared from soluble fraction S_{225} or from crude or washed ribosomes. Preparations were treated under vacuum with 6 N HCl at 110°C for 3 h. Hydrolysates were dried then dissolved in 2.5 % formic acid/ 7.8 % acetic acid at pH 1.9, and subjected to paper electrophoresis for 120-150 min under 2 000 volts (22) together with authentic phosphothreonine and phosphoserine samples. Amino acids were revealed with 0.5 % ninhydrin in acetone. Radioactive spots were detected after autoradiography of electrophoregrams, then cut out and counted in a Packard model 3380 spectrometer. In each case, the relative proportion of labeled phosphothreonine and phosphoserine was calculated. In some instances, proteins were first separated by gel electrophoresis and extracted individually with 1 % SDS/6 M urea before acid hydrolysis and phosphoamino acid analysis.

RESULTS

Cells were grown in the presence of radioactive orthophosphate, then soluble and ribosomal fractions were extracted and subjected to SDS-gel electrophoresis and autoradiography. The results obtained in a typical analysis of the soluble fraction S_{225} are presented in Fig. 1 (b and c). It can be seen that four major bands, P_1 to P_4, are labeled to a significant extent and that several other faint bands are also revealed. All of those ^{32}P-containing species appear to correspond to protein molecules. Indeed, besides their specific staining with Coomassie blue, they were

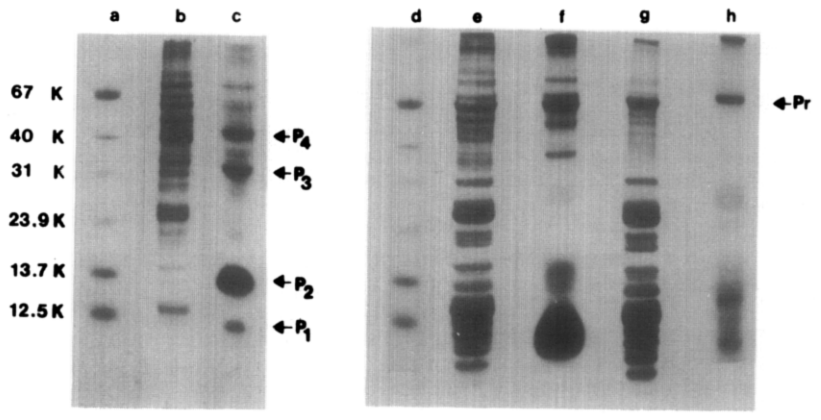

Fig. 1. Analysis of phosphorylated proteins of *E. coli*. Cells were grown in presence of [^{32}P] orthophosphate, and proteins from the soluble and ribosomal fractions were prepared and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography as described in Methods. b, e and g are the Coomassie blue-stained proteins from respectively soluble fraction S_{225}, crude ribosomes and washed ribosomes. c, f and h are the autoradiograms corresponding to the same fractions respectively. The following reference proteins (a and d) were analyzed simultaneously and stained : bovine serum albumin (67 K), aldolase (40 K), deoxyribonuclease (31 K), trypsin (23.9 K), ribonuclease A (13.7 K) and cytochrom C (12.5 K).

found to be resistant to ribonuclease treatment whereas they were completely hydrolyzed by pronase.

When proteins extracted from crude ribosomes were analyzed under similar conditions (Fig. 1, e and f), one main radioactive band and at least five other well-defined bands were detected in the zone of high molecular weight compounds. Also, a large fast-moving spot was revealed. This spot appeared to be insensitive to ribonuclease action and also to pronase treatment (not shown here), the latter observation suggesting that it does not correspond to any polypeptide entity. In contrast, all other bands were resistant to ribonuclease but were digested by pronase, like proteins from the soluble fraction.

The analysis of proteins extracted from washed ribosomes confirmed first (Fig. 1, g) that incubation with 1 M NH_4Cl induces the release of several large proteins initially adsorbed on crude

particles and that are usually not classified as "true" ribosomal proteins (15, 23). Those of them which were radioactively labeled have therefore disappeared on the corresponding autoradiogram (Fig. 1, h). Also, it can be noted that the fast-moving spot mentioned above is not longer visible. However, of special interest is the fact that one high-molecular weight band, Pr, is still clearly detected and that two low-molecular weight bands also seem to be labeled although to a much lesser extent. Here again, these bands were found to be ribonuclease-resistant but pronase-sensitive.

In order to determine the nature of the bound radioactive moiety, two series of experiments were carried out. In the first one, the sensitivity of the various protein preparations to alkaline phosphatase was checked out. The results then obtained showed clearly that, in all cases, the radioactive material was completely removed after such enzyme treatment since no more labeled bands could be detected on autoradiograms. In the second series of experiments, total proteins from the soluble fraction and proteins from crude or washed ribosomes were subjected to acid hydrolysis, and the labeled material thus liberated was analyzed by paper electrophoresis. In addition, a similar treatment was applied individually to each of the two most labeled proteins, P_2 and P_3, after migration and selective extraction from polyacrylamide gels. In all cases, the radioactive hydrolyzed material was found to co-migrate with phosphothreonine and phosphoserine standards. The data presented in Table 1 show, however, that the relative proportion of these modified amino acids depends on the type of protein. Indeed, total or individual soluble proteins contain a high percentage of phosphoserine whereas proteins from washed ribosomes are particularly rich in phosphothreonine. Intermediate values are obtained for proteins prepared from crude ribosomes,

Table 1 - Relative proportion of phosphothreonine and phosphoserine in E. coli proteins.

Phosphoamino acid	Protein				
	soluble	P_2	P_3	crude ribosomes	washed ribosomes
Phosphothreonine (%)	13	10	18	51	78
Phosphoserine (%)	87	90	82	49	22

Total or individual soluble proteins and total proteins from crude or washed ribosomes were hydrolyzed with 6N HCl for 3 h at 110°C. Hydrolysates were then analyzed by paper electrophoresis and the relative percentage of radioactive phosphothreonine and phosphoserine was determined as described in Methods.

probably due to the fact that such preparation is a mixture of soluble and true ribosomal proteins. It is worth mentioning, moreover, that neither modified amino acid was detected upon individual analysis of the radioactive fast-moving spot described above, which confirms that this compound is most likely not a protein.

DISCUSSION

The results here presented provide strong support for the concept that a protein kinase activity exists in E. coli which phosphorylates several proteins from the soluble and ribosomal fractions of the cell.

Under the experimental conditions used in this study, any possible contamination of proteins by polyphosphates (5, 6) can be excluded since all polyacrylamide gels were systematically treated with hot trichloroacetic acid before any further analysis. Moreover, the presence of acylphosphates (24, 25) in the radioactive material can also be ruled out because all preparations were found to be sensitive to alkaline phosphatase. Finally, the finding of phosphothreonine and phosphoserine in acid hydrolysates of proteins indicates that a covalent modification occurred as a

result of protein kinase reaction rather than the formation of phosphorylated intermediates in enzymatic mechanisms. Indeed, it has been shown previously that most of such intermediates of kinase reactions are either carboxylphosphates (24) or histidylphosphates (26).

The exact nature of phosphorylated *E. coli* proteins remains to be determined. The only information so far available is that the major soluble proteins, P_1 to P_4, possess an apparent molecular weight of about 12 000, 13 000, 35 000 and 49 000 daltons respectively, as calculated by comparison with marker proteins. In the ribosomal fraction, the major protein Pr has an approximate molecular weight of 68 000 daltons and might be therefore identical to protein S_1 from the small 30S ribosome subunit (27) or to the recently described protein A (28).

The fact that the proportions of phosphoamino acids are different in soluble and ribosomal proteins may reflect merely that these two classes of proteins have a different threonine-serine content and/or a different conformational state which generates a variable accessibility to phosphorylation. Alternatively, one may envisage that the protein kinase activity of *E. coli* is due, in fact, to multiple enzymes with a different protein substrate specificity. Further experiments, involving namely enzyme purification, are required to decide between those two hypotheses. They are currently in progress in our laboratory.

In any case, since it appears that *E. coli* like *S. typhimurium* (5) harbor protein kinase activities, it seems now reasonable to expect that, by analogy with eucaryotic systems (11), protein phosphorylation reactions may also play a role in the regulation of cellular function in procaryotes.

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DANSYLATION OF BACTERIORHODOPSIN NEAR THE RETINAL ATTACHMENT SITE

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SUMMARY: The purple membrane of Halobacterium halobium was reacted with 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl chloride) at pH 8.0. Chromophoric and functional properties of the product appear unaltered. Approximately 2 moles of dansyl group were incorporated per mole of bacteriorhodopsin, part bound to bacteriorhodopsin and part bound to lipids. Purification and fragmentation of the protein showed most of the dansyl modification in a fragment containing residues 33 to 56. Amino acid analysis indicates that the major dansylated site is lysine 40. We conclude that, contrary to published models, 1) bacteriorhodopsin folds in a way that exposes lysine 40 at the membrane surface, and 2) this side chain is not involved in the proton pump mechanism.

The purple membrane of Halobacterium halobium acts as a light driven proton pump (1). The membrane occurs naturally as a two dimensional crystal (2) and contains only a single retinyl protein, bacteriorhodopsin (3). The crystal structure (4) and amino acid sequence analyses (5,6) have provided a framework for understanding the molecular mechanism of the purple membrane proton pump. The 7 Å resolution crystal structure showed that bacteriorhodopsin is largely folded into seven regions of α-helix (4). However, the connection scheme of the helices is unknown. The amino acid sequence has suggested several possible folding patterns (5,7). While a detailed structural model is not yet available, much attention has focused on spectroscopic methods to elucidate the proton pump mechanism. In particular, resonance Raman (8) spectroscopy points to the involvement of certain amino acid side chains in pump activity. A proposed mechanism is based on this evidence (8).

We have been examining various chemical derivatives of the purple membrane as a means of studying the proton pump mechanism (9). We now report the reaction of the purple membrane with dansyl chloride[3]. The major site of modifica-

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3. Abbreviations: Dansyl: 5-dimethylaminonaphthalene-1-sulfonyl; HPLC: high performance liquid chromatography

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