Two-dimensional analysis of proteins phosphorylated in *E. coli* cells

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Proteins phosphorylated in *Escherichia coli* cells were analyzed by the O'Farrell two-dimensional gel technique. Cytoplasmic and ribosomal fractions were studied separately. Double labeling with [^{32}P]orthophosphate and [^{35}S]sulfate followed by selective autoradiographic detection of each radioisotope allowed precise location of 12 major phosphoproteins on the total protein pattern of bacteria. Both the molecular mass and isoelectric point of these phosphoproteins were determined.

Protein phosphorylation *Bacterial protein kinase* *Two-dimensional analysis*
Cytoplasmic phosphoprotein *Ribosome* Escherichia coli

1. INTRODUCTION

In recent years, conclusive evidence has shown that not only eukaryotes but also prokaryotes contain a protein kinase activity [1,2]. In *E. coli* cells, it has been shown that this activity can phosphorylate several proteins at the level of serine, tyrosine, and, to a lesser extent, threonine residues [3,4]. However, little is known so far on the nature, and even on the number, of these phosphorylated proteins. In fact, only one of them has clearly been identified as yet: the NADP-dependent isocitrate dehydrogenase whose reversible phosphorylation is connected with the partition of carbon flux between the Krebs cycle and the glyoxylate bypass [5,6].

Therefore, in the present work, an attempt was made to characterize further the phosphoproteins of *E. coli*. For this purpose, the two-dimensional gel technique as in [7] was used, since it has a much higher resolving power than the one-dimensional separating systems generally utilized in [3,4]. The results reported here mainly show that 10 major phosphorylated proteins can be detected specifically in the cytoplasmic fraction of bacteria, and one in the ribosomal fraction. One additional protein is found simultaneously in both fractions. A catalogue of the isoelectric point and molecular weight of these various phosphoproteins is presented.

2. EXPERIMENTAL

2.1. *Strain and culture medium*

The previously described [8] strain of *E. coli* (*arg, leu, thr, his, thi*) was used. Cells were cultured at 37°C in a low-phosphate minimal medium [4] containing 25 mM acetate as the carbon source, and supplemented with the 4 essential amino acids (80 μg/ml each) and the vitamin (5 μg/ml). Radioactive [^{32}P]orthophosphate (40 μCi/ml) and [^{35}S]sulfate (150 μCi/ml) were added at the beginning of the exponential phase of growth. After 2–3 generations, cells were harvested by low-speed centrifugation.

2.2. *Preparation of cellular extracts*

Bacteria were suspended in a buffer containing 10 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$ and 50 μg/ml pancreatic ribonuclease, and disrupted by repeated ultrasonic treatment. The sonicated extract was stirred for 15 min at 4°C in the presence of 50 μg/ml pancreatic deoxyribonuclease then treated with 0.12 vol of a 3% sodium

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dodecylsulfate/10% β-mercaptoethanol mixture as in [9]. After centrifugation for 25 min at 30000 × g the liquid layer (fraction S30) was collected. In some experiments, fraction S30 was further centrifuged for 180 min at 225000 × g to sediment the crude ribosomes. When required, the ribosomal pellet was resuspended at a final concentration of about 10 mg/ml in a buffer at pH 7.6 containing 50 mM Tris, 10 mM $MgCl_2$ and 1 M NH_4Cl, and maintained overnight at 4°C [4]. Salt-washed ribosomes thus prepared were pelleted by centrifugation at 225000 × g for 180 min.

2.3. *Preparation of protein fractions*

Proteins from fraction S30 were obtained by overnight precipitation with 5 vols of 95% acetone at −20°C. They were collected by low-speed centrifugation and dried under vacuum.

Ribosomal proteins were extracted from crude or salt-washed ribosomes by the acetic acid procedure [10]. Briefly, ribosomes were suspended in 1 vol. of 10 mM Tris–HCl (pH 7.7)/100 mM magnesium acetate, and treated for 60 min at 4°C with 2 vols of 17 M acetic acid. Proteins thus solubilized were dialyzed against 1 M acetic acid then lyophilized.

Protein concentration in various preparations was determined spectrophotometrically after staining with 0.01% Coomassie blue G250 in 4.7% ethanol/8.5% phosphoric acid according to [11].

2.4. *Two-dimensional separation of proteins*

Proteins of fraction S30 (20–40 μg) and ribosomal proteins (180–200 μg) were analyzed by the O'Farrell gel technique [7]. First-dimension isoelectric focusing to equilibrium (7000 V·h) was carried out in 4% acrylamide and pH 3 to 10 ampholine in the presence of 9.5 M urea. Electrophoresis in the second dimension was performed in 11.4% acrylamide and 0.1% sodium dodecylsulfate for 7 h under 300 V. After protein migration, gels were soaked in 16% trichloroacetic acid and heated for 45 min at 95°C. They were then incubated for 120 min at room temperature in 7.5% acetic acid/30% methanol, dried under vacuum and autoradiographed.

2.5. *Autoradiography*

The autoradiographic detection of double-labeled (^{35}S and ^{32}P) proteins on gels was made according to [12]. Two films were exposed simultaneously for each gel; one recorded directly the β emissions from ^{35}S and the other, shielded by an aluminum foil, recorded scintillation photons from an intensifying screen excited by the ^{32}P emissions. Autoradiography was performed at low temperature (−80°C) so that there was no detection of ^{35}S on the ^{32}P film, and only a few percent of the β emissions detected on the ^{32}P film exposed the ^{35}S film. In these conditions, discrimination of the two isotopes was virtually complete.

3. RESULTS

Bacteria were grown on acetate in the presence of both [^{35}S]sulfate and [^{32}P]orthophosphate, cellular fraction S30 was prepared and proteins

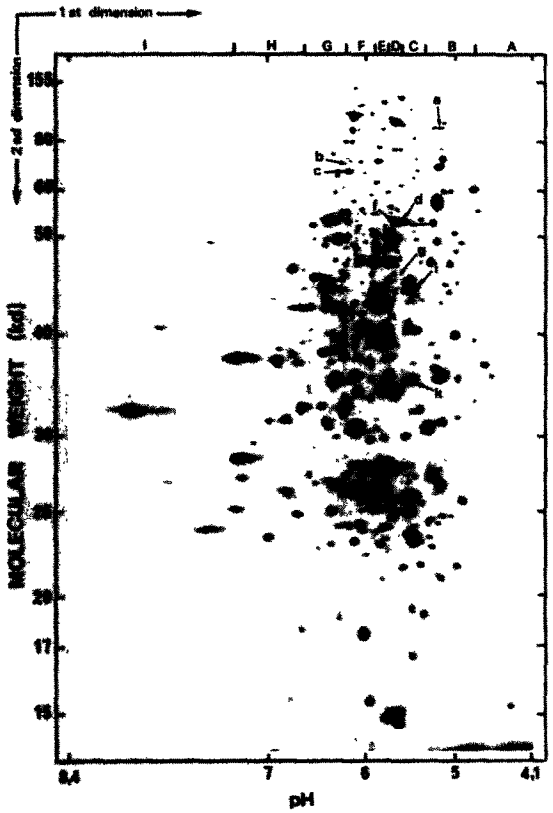

Fig.1. Autoradiography of ^{35}S-labeled proteins from *E. coli* fraction S30. Proteins were separated by the O'Farrell two-dimensional gel technique and autoradiographed for 48 h. The location of phosphorylated proteins (see fig.2) is indicated by arrows.

were analyzed by the two-dimensional gel technique [7]. A typical autoradiogram of ^{35}S-labeled proteins is presented in fig.1. It is quite similar to that described by Neidhardt et al. [13]. Phosphorylated proteins were specifically revealed by selective autoradiography of ^{32}P-labeled molecules [12]. As shown in fig.2, 11 major radioactive proteins (a to k) could thus be detected. The precise location of these phosphorylated proteins on the total protein pattern was achieved (fig.1) by superimposing the original ^{32}P- and ^{35}S-autoradiograms, both obtained from the same two-dimensional gel in each analysis.

Since it is known [11] that ribosomal proteins are not extracted when preparing cellular fraction S30 (except for the very low proportion of those [14] which are present in bacteria in a free soluble state), they were purified separately from isolated ribosomes under conditions yielding their complete solubilization [10] then subjected to two-dimensional analysis as above. The results in fig.3 show that crude ribosomes (upper diagram) contain 2 major phosphorylated proteins (d and d'). However, only protein d is still present in ribosomes after extensive washing with concentrated ammonium chloride (lower diagram). It can be noted that this protein was already detected in fraction S30 (fig.2), whereas surprisingly protein d' was visible only in the ribosomal fraction.

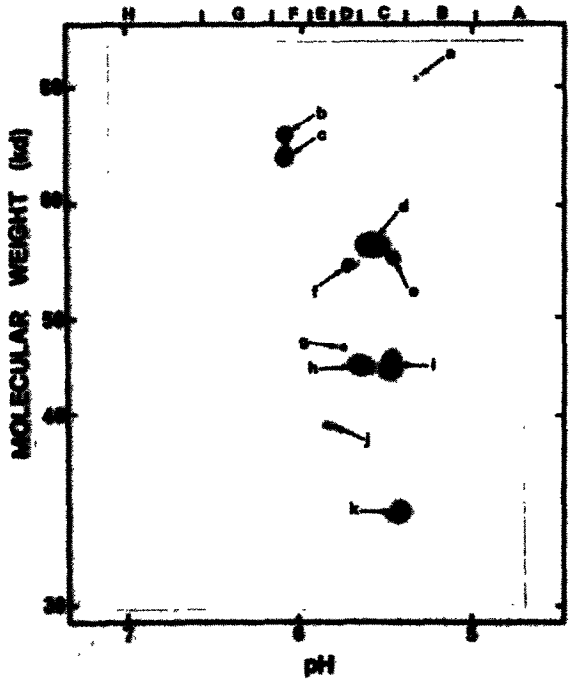

Fig.2. Autoradiography of ^{32}P-labeled proteins from fraction S30. Radioactive phosphoproteins (a to k) were detected by selective autoradiography according to [12]. Their relative location on total protein pattern is indicated in fig.1.

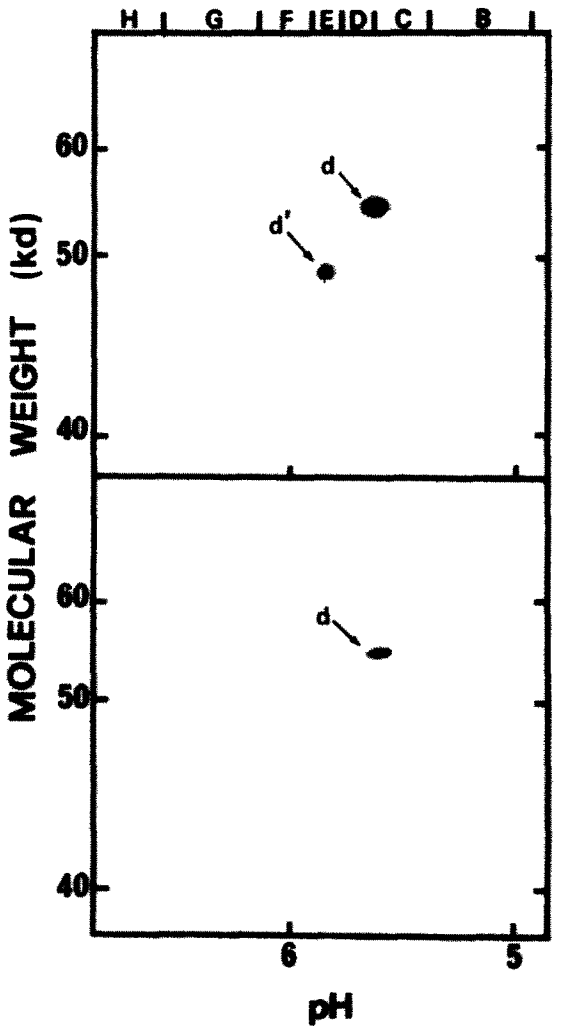

Fig.3. Autoradiography of ^{32}P-labeled proteins from ribosomes. Proteins were extracted from either crude ribosomes (upper diagram) or ribosomes previously washed with 1 M NH_4Cl (lower diagram), and analyzed as in fig.2.

The analytical gel system used here allowed, for any resolved protein, the determination of both its isoelectric point and molecular mass. These two parameters were therefore measured for the

Table 1

Molecular mass and isoelectric point of major *E. coli* phosphoproteins

Protein	Protein fraction	Molecular mass (kDa)	Isoelectric point
a	C	83.0	5.25
b	C	74.0	6.1
c	C	72.0	6.1
d	C,R	54.5	5.6
d′	R	48.0	5.75
e	C	54.1	5.55
f	C	54.0	5.65
g	C	45.4	5.65
h	C	44.4	5.6
i	C	44.0	5.3
j	C	39.8	5.75
k	C	35.0	5.3

For each phosphoprotein extracted from either the cytoplasmic fraction (C) or the ribosomes (R), the molecular mass and isoelectric point were determined, under denaturing conditions, from its location on O'Farrell gel as detected by autoradiography

various phosphorylated proteins. The corresponding values are listed in table 1.

4. DISCUSSION

E. coli cells contain 12 major phosphorylated proteins: 10 are found only in the cytoplasmic fraction, 1 is present specifically in the ribosomal fraction, and 1 is detected in both fractions. It is not clear yet whether this latter protein (protein d) is a cytoplasmic protein adventitiously, but firmly, adsorbed on the surface of ribosomes or a ribosomal protein partially released from the organelles.

The resolving power of the O'Farrell technique is very high and the probability of two unrelated polypeptides occupying exactly the same position on this type of gel has been found negligible [15]. Nevertheless, it cannot be totally excluded that two different phosphorylated proteins, or more, may comigrate and appear in the same radioactive spot. Also, it is possible that some faint radioactive proteins could not be revealed under our experimental procedure. Moreover, any very acidic (pHi < 4.1) or basic (pHi > 8.4) protein should have escaped our analysis performed under equilibrium conditions in the first-dimension isoelectric focusing. It is therefore likely that 12 is the minimal number of phosphoproteins in *E. coli*. However, this number will in any case remain relatively very low whenever compared to the total of 1100 different proteins that have been resolved from bacterial extracts on O'Farrell gels [13]; this clearly indicates that protein phosphorylation is a rather highly specific process in bacteria.

Of special interest is the fact that only proteins phosphorylated through the formation of phosphate ester bonds catalyzed by a protein kinase activity were analyzed in this work. Indeed, all other phosphate-containing proteins modified, for instance, at acyl or amino groups such as certain phosphorylated enzyme intermediates [16] could not be visualized since they were dephosphorylated during the hot trichloroacetic acid treatment systematically applied to gels before autoradiography [17]. Such treatment led, in addition, to the elimination of polyphosphate contaminants [18].

The catalogue in table 1 is, to our knowledge, the first so far available for *E. coli* phosphorylated proteins. Although it might be incomplete for the reasons mentioned above, it nevertheless brings an improved characterization of phosphoproteins over previous reports [3,4]. Obviously, the next step should consist in identifying these proteins. From this point of view, the comparative analysis of their location on two-dimensional autoradiograms and that of the 164 proteins already identified [13] can be expected to provide useful information. Thus, by referring to its relative position on gels (and also to its physical properties described by others [5] and its enhanced phosphorylation on acetate medium [5,6]), it seems likely that protein i is identical to the NADP-dependent isocitrate dehydrogenase. The identification of the other phosphorylated proteins is in progress.

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