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FEMS Microbiology Letters 138 (1996) 129–134

FEMS MICROBIOLOGY LETTERS

Phosphorylation of GroEL, DnaK and other proteins from *Thiobacillus ferrooxidans* grown under different conditions

Michael Seeger, Gonzalo Osorio, Carlos A. Jerez *

Departamento de Bioquímica, Facultad de Medicina, Universidad de Chile, Casilla 70086, Santiago-7, Chile

Received 18 December 1995; revised 5 February 1996; accepted 14 February 1996

Abstract

The levels of phosphorylation of the chaperones DnaK and GroEL and other proteins varied when cells of *Thiobacillus ferrooxidans* were subjected to phosphate starvation. The phosphorylated amino acid of GroEL was found to be threonine. Our results show that not only heat shock, but also a nutrient starvation stress leads to phosphorylation of chaperones and, in addition, support the possible role of phosphorylation of these proteins in the sensing and regulation of stress responses in bacteria.

Keywords: DnaK; GroEL; *Thiobacillus ferrooxidans*; Chaperone phosphorylation; Stress response and protein phosphorylation

1. Introduction

Thiobacillus ferrooxidans is a chemolithotrophic microorganism capable of using ferrous ions and reduced sulfur compounds as energy sources [1]. This microorganism has an important role in the bioleaching of minerals [1,2]. During this process, the bacteria are normally subjected to several stressing conditions, such as temperature changes, lack of nutrients or pH changes, which may affect the efficiency of the bacterial action. We have been studying the sensory and adaptive responses of these microorganisms to phosphate starvation [3,4], heat shock [5] and pH changes [6].

When bacteria such as *Escherichia coli* are exposed to heat shock and several other stressful conditions, they respond by inducing the synthesis of several proteins known as stress proteins, some of which are molecular chaperones. These include the molecular chaperones DnaK and GroEL ([7] and references therein). These two proteins protect the cell against stressing injuries because they prevent the aggregation of the damaged proteins and mediate their refolding to the native state. Members of the chaperone families are also expressed in normally growing cells, facilitating the transport of proteins across cell membranes, cotranslational protein folding, proper protein assembly and other normal functions of cells [7]. The regulation of the bacterial chaperones is mainly based on their transcriptional induction by stressful conditions. In addition, *E. coli* DnaK possesses an autophosphorylating activity [8], and it has been recently described that the chaperones DnaK and GroEL are also regulated by phosphorylation [9,10]. This modification markedly improves the capacity of DnaK to function as a molecular chaperone, enhancing its binding to unfolded

* Corresponding author. Tel./Fax: +56 (2) 735 6384.

PII S0378-1097(96)00084-5

proteins. The phosphorylated GroEL also changes its properties, becoming more active in promoting the refolding of damaged proteins, being capable of working in a GroES-independent manner [10,11]. Phosphorylation of both GroEL and DnaK could be a critical adaptation which confers resistance to heat shock [11].

We have previously studied the heat shock response and identified and characterized DnaK and GroEL in *T. ferrooxidans* [12]. Here, we demonstrated that when *T. ferrooxidans* is grown in ferrous iron medium under normal conditions, both GroEL and DnaK are phosphorylated in vivo. When the bacterium is subjected to lack of phosphate or a shift in oxidizable substrate, not only the two chaperones, but several other proteins are also modified in their degree of phosphorylation.

2. Materials and methods

2.1. Bacterial strain and growth conditions

The chemolithoautotrophic microorganism employed in this study was *T. ferrooxidans* strain R2 [6]. It was grown at 30°C and at pH 2.5 in a modified ferrous ion-containing medium that contained 0.04 g K_2HPO_4, 33.3 g $FeSO_4 \cdot 7H_2O$, 0.4 g $MgSO_4 \cdot 7H_2O$ and 0.1 g $(NH_4)_2SO_4$ per liter and no trace metals [6]. Growth under phosphate-starvation conditions was in the same medium, except that the phosphate salt was omitted. To have phosphate-starvation effects, a second transfer to the medium without phosphate was necessary [4]. Growth in sulfur as energy source was in the same medium at pH 2.5 but replacing the ferrous ion by sulfur prills.

2.2. Labeling of T. ferrooxidans proteins in vivo

For the radioactive labeling of bacterial cells with $H_3{}^{32}PO_4$, 4×10^{10} cells were suspended in 0.5 ml of the medium described above, but with the amount of phosphate reduced to 1/100 in the unstarved control or with no phosphate in the phosphate-starved cells. Reducing the phosphate in the control cells avoided the dilution of the radioactive label. Under these conditions, we obtained the same growth and 2-D PAGE protein pattern as that observed with the normal medium. Cells grown in ferrous iron or sulfur under normal conditions were incubated with 400 μCi of $H_3{}^{32}PO_4$ for 15 h. Cells starved for phosphate were incubated with 100 μCi of $H_3{}^{32}PO_4$ for 15 h. The total radioactivity incorporated into proteins was determined by scintillation counting of the corresponding aliquots.

2.3. 2-D PAGE analysis

Total cell proteins were separated by two-dimensional polyacrylamide gel electrophoresis with nonequilibrium pH gradient (NEPHGE) as described previously [6], employing ampholites (pH 3–10) from Bio-Rad Laboratories. The cell samples (3.5 mg wet weight of unlabelled cells, or 200 000 cpm contained in cells labeled by growth in $H_3{}^{32}PO_4$) were resuspended in 100 μl of sonication buffer (10 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$ and 50 μg of pancreatic RNAase per ml), sonicated and treated with DNAase (50 μg per ml, final concentration). The mixture was then lyophilized and dissolved in lysis buffer as described previously [6]. Molecular mass standards for the second dimension were from Bio-Rad laboratories. The relative intensity of the autoradiographic spots from phosphorylated proteins separated by 2-D NEPHGE were determined by using a Hewlett-Packard Scan Jet Plus device and the GelPerfect image analysis program (kindly provided by S. Bozzo, Universidad de Chile).

2.4. Analysis of phosphoamino acids

To determine the nature of phosphoamino acids, radioactively phosphorylated GroEL was recovered from Coomassie brilliant blue-stained NEPHGE gels by excising the protein spot (five spots from the corresponding gels). The gel pieces were concentrated by placing them in a gel slot of a new SDS-slab gel [12]. After the electrophoretic run, the protein was electroblotted onto polyvinylidene difluoride (PVDF) membranes and subjected to hydrolysis in the presence of 6 M HCl at 110°C for 1.5 h. Hydrolyzed amino acids were separated by thin layer chromatography, employing the buffer 5:3 isobutyric acid:0.5 M ammonium hydroxide [13], and using as standards phosphotyrosine, phosphothreonine and phosphoserine (Sigma Chemical Co.).

3. Results and discussion

3.1. Pattern of protein phosphorylation in T. ferrooxidans grown in ferrous iron as oxidizable substrate

Total proteins of *Thiobacillus ferrooxidans* grown with ferrous ion as energy source were separated by 2-D NEPHGE [6], stained with Coomassie brilliant blue, and used as a reference to locate the coordinates of the phosphoproteins studied (Fig. 1). Fig. 2 shows the in vivo phosphorylation pattern of *T. ferrooxidans* subjected to different conditions. Several phosphorylated proteins with varying degrees of phosphorylation could be detected. Most of the phosphoproteins were located in the pH 5–7 range of the gel, as observed for the phosphoproteins detected in *E. coli* [14]. The majority of the phosphoproteins of *T. ferrooxidans* corresponded to stained spots in the 2-D gel of Fig. 1 (see for example spots g, d, 20, 21, x and y). Other polypeptides could be detected only by their radioactivity (for example, spots a, b, m, l, n), suggesting that they are poorly stained with Coomassie brilliant blue or that they are normally present in much lower concentrations in the bacterial cell.

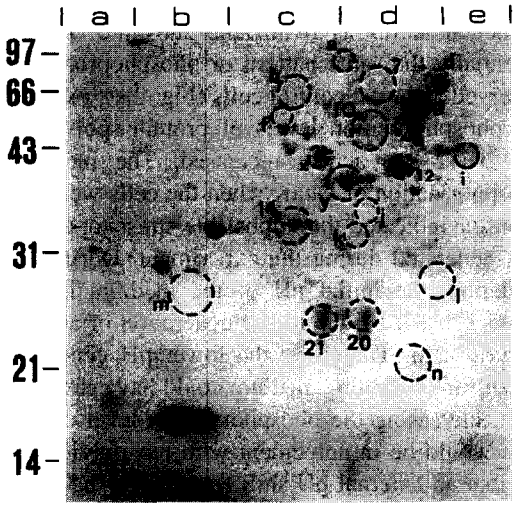

Fig. 1. Two-dimensional separation of proteins from *T. ferrooxidans*. Total proteins from *T. ferrooxidans* grown in ferrous iron were separated by 2-D NEPHGE and were stained with Coomassie brilliant blue. Protein spots enclosed by circles correspond to the proteins phosphorylated under different conditions seen in Fig. 2. The numbering was done arbitrarily. Top letters designations from a to e indicate zones of decreasing pH: a, 10–9; b, 9–7; c, 7–5.8; d, 5.8–4.8; e, 4.8–3.0. Numbers to the left of the gels indicate the molecular mass markers in kDa.

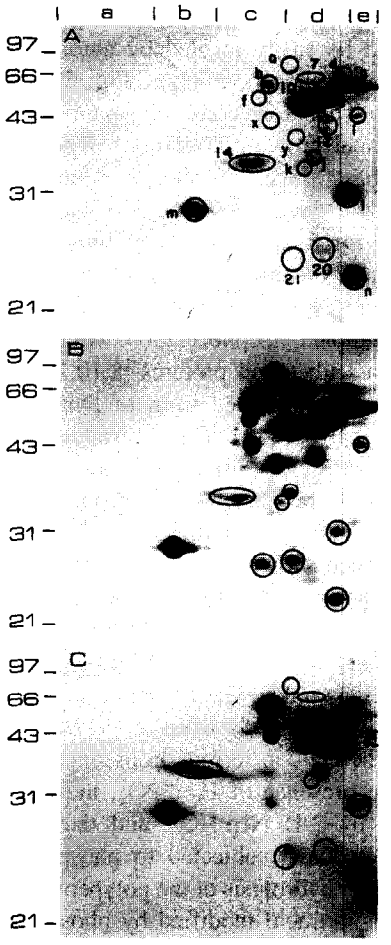

Fig. 2. Global pattern of phosphorylation of total proteins from *T. ferrooxidans* grown under different conditions. *T. ferrooxidans* was grown in the presence of $H_3{}^{32}PO_4$ and in ferrous iron (A, B) under normal conditions (A), without phosphate (B) or in the presence of phosphate but with sulfur as oxidizable substrate (C). The proteins were separated by 2-D NEPHGE and stained with Coomassie brilliant blue followed by autoradiography. Protein spots were named as in Fig. 1. Numbers to the left of the gels indicate the molecular weight markers in kDa.

When *E. coli* is grown in different carbon sources, similar phosphorylation patterns are obtained [14]. When *T. ferrooxidans* was grown in ferrous iron (Fig. 2A), 13 polypeptides were phosphorylated to different extents. The 2-D coordinates of proteins d (DnaK) and g (GroEL) from *T. ferrooxidans* were identified previously by Western-blotting and N-

terminal end sequencing [12] (see Fig. 1). Therefore, they could be easily located by autoradiography of the separated phosphoproteins. Fig. 2A reveals that these two chaperones are phosphorylated in vivo under normal growing conditions. Consequently, an in vivo basal level of phosphorylation would be necessary for the proper actions of these chaperones in *T. ferrooxidans*. It has been previously demonstrated in *E. coli* that under stressing conditions such as heat shock, a fraction of DnaK and GroEL undergoes reversible phosphorylation, which alters their interaction with target proteins [8,10,11]. Moreover, only the phosphorylated form of DnaK could bind unfolded peptides [11]. These results suggested that under non-stress conditions a fraction of DnaK and GroEL should be phosphorylated. Our results are in agreement with these findings.

It has been shown in *E. coli* that DnaK is phosphorylated normally at serine, whereas it is modified essentially at threonine after M13 phage infection of the cells [15]. The amino acid phosphorylated in *E. coli* GroEL is unknown. To determine the modified amino acid in GroEL from *T. ferrooxidans*, after growing the cells in ferrous iron-containing medium and in the presence of $H_3{}^{32}PO_4$, the proteins were separated by 2-D NEPHGE and the GroEL spots were isolated and subjected to phosphoamino acid analysis after hydrolysis of the polypeptide. We found that the amino acid modified by phosphorylation in GroEL was threonine (results not shown).

3.2. Phosphate starvation changes the pattern of phosphoproteins in T. ferrooxidans

The phosphate ion is an essential nutrient for all living organisms. Therefore, the lack of this compound is a stressing condition for microorganisms, inducing the stress response. When *T. ferrooxidans* was subjected to lack of phosphate, several proteins increased their phosphorylation levels, noticeably spots a, b, e, i, y, x, 12, 20 and 21, and also DnaK and GroEL (Fig. 2B). One of the most evident changes was the great increase in the phosphorylation level of DnaK (compare Fig. 2A with 2B). Not all the observed variations in phosphorylation can be attributed to a change in the phosphorylation process per se, but could instead be the consequence of larger numbers of phosphorylated molecules synthesized. In *T. ferrooxidans*, we detected previously several changes in the levels of proteins synthesized after phosphate starvation [3]. Of the phosphorylated proteins seen in Fig. 2B, and which are stainable by Coomassie brilliant blue (Fig. 1), spots i, x, 20, 21 and possibly a, b and y showed an increased synthesis after phosphate starvation. Under the same conditions, spots e, d (DnaK) and g (GroEL) did not greatly change and spot 12 showed decreased synthesis. The changes in phosphorylation of DnaK and GroEL under our conditions therefore appear to be changes in the phosphorylation per se. A similar finding has been observed for the phosphorylation of DnaK from *E. coli*, whose phosphorylation strongly increased under phage M13 infection, while its rate of synthesis was not significantly changed [15]. On the other hand, in *E. coli*, Matin observed the induction of GroEL synthesis upon 30 min of phosphate starvation whereas DnaK synthesis was not induced under this condition [16].

3.3. Protein phosphorylation in T. ferrooxidans grown in sulfur as oxidizable substrate

When the bacteria were grown in the presence of elemental sulfur as oxidizable substrate (Fig. 2C), essentially the same pattern of phosphoproteins was observed as in the control cells (Fig. 2A), except that the phosphorylation levels of protein spots b, d, f, 12, 20, 21, x and y increased. The increase in phosphorylation of DnaK when the cells were grown in sulfur indicated that a possible stressing condition was generated during the experiment. During sulfur oxidation, the initial pH of the medium decreases (from 2.5 to 1.5) due to the generation of additional sulfuric acid. Therefore, the lower pH generated in the sulfur-containing medium could have stressed the cells, increasing the phosphorylation level of DnaK. This would be in agreement with our previous finding that an external pH shift from 3.5 to 1.5 induced in *T. ferrooxidans* the synthesis of several heat-shock-like proteins [6]. When we analyzed the proteins synthesized by *T. ferrooxidans* grown on ferrous iron or elemental sulfur as energy sources, we found that phosphorylated spots 14 and x showed reduced synthesis in sulfur-grown cells compared with ferrous iron-grown cells, and DnaK (spot d) synthesis increased two-fold (unpublished results).

Table 1
Changes in the phosphorylation of proteins from *T. ferrooxidans* grown under different conditions

Spot	Molecular mass (kDa)	Phosphorylation levels relative to control	
		−Pi	Sulfur grown
a	76	++++	nc
b	65	++++	++++
d	68	++++	+++
e	57	++	++
f	60	++++	++++
g	62	++	nc
i	52	++	nc
j	40	+	+
k	38	++	+
l	33	(−)	(−)
m	30	nc	nc
n	23	(−)	(−)
x	52	++++	++++
y	38	++++	++++
7	67	++++	nc
10	55	nc	nc
12	43	+++	++++
14	36	nc	nc
20	28	++++	+++
21	27	++++	+++

The changes in intensity of the phosphoprotein spots relative to the control cells are indicated as a very intense increase (++++), intense increase (+++), moderate increase (++), low increase (+), decrease (−) or without important change (nc). These variations were estimated from the autoradiograms shown in Fig. 2. The approximate molecular masses were determined measuring the relative migration distances in the second dimension of the gel in Fig. 1. Control cells were grown in ferrous iron medium.

The phosphorylation of DnaK showed an estimated increase of four-fold under the same condition, suggesting accordingly that this modification is a phosphorylation per se. Whether these phosphorylations are associated with proteins involved in the specific oxidation of sulfur in *T. ferrooxidans* is not known at present.

A summary of the changes in the principal phosphoproteins analyzed in *T. ferrooxidans* is shown in Table 1. It is clear that the changes in intensity of the phosphorylated spot of DnaK were much greater than those seen for GroEL under the conditions studied. The variations in phosphorylation of DnaK under phosphate starvation and elemental sulfur as energy source are changes per se in the phosphorylation of this protein. The findings of McCarty and Walker [9,17] and those of Sherman and Goldberg [11] demonstrated an important aspect of the heat shock response. That is, in addition to the changes in gene transcription at high temperature, there is also a posttranslational mechanism of regulation of this response, based upon reversible phosphorylation [11]. Different stress responses may be regulated in diverse ways, one of them involving induction of the expression of stress proteins, which is a high energy-requiring event. Under a stress such as phosphate starvation, an alternative or additional regulation could be posttranslational modification of a fraction of the stress proteins or a combination of both events. The physiological relevance of such a posttranslational mechanism could be related to the saving of energy by stressed cells depleted of energy sources, since such a regulatory mechanism would be energetically less expensive for the cell.

Our results support the idea that the phosphorylation of the chaperones DnaK and GroEL could be part of a general response to stress conditions, and not only for the heat shock response, since when *T. ferrooxidans* was exposed to a non-heat-shock stress, such as phosphate starvation, DnaK and GroEL showed increased phosphorylation levels. The increase of the phosphorylation per se of DnaK under different stressful conditions suggests that this chaperone could directly sense not only the temperature [17], but also several stressing environments, being a 'stress sensor'.

Acknowledgements

Supported by grants from FONDECYT 194/0379, Fondecyt 2950019, UNIDO 91/049 and SAREC. We thank Marta Gatica for her kind advice with the determination of phosphorylated amino acids and Catherine Connelly for her assistance with the language.

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