Protein Phosphorylation in Response to Stress in Clostridium acetobutylicum

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The possible involvement of protein phosphorylation in the clostridial stress response was investigated by radioactively labeling growing cells of Clostridium acetobutylicum with $^{32}$P, or cell extracts with [$\gamma$-32P]ATP. Several phosphoproteins were identified; these were not affected by the growth stage of the culture. Although the extent of protein phosphorylation was increased by heat stress, the phosphoproteins did not correspond to known stress proteins seen in one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Purified clostridial DnaK, a stress protein, acted as a kinase catalyzing the phosphorylation of a 50-kilodalton protein. The phosphorylation of this protein was enhanced in extracts prepared from heat-stressed cells. Diadenosine-5',5''-P',P'-tetraphosphate had no influence on protein phosphorylation.

A number of functions in procaryotes, notably the responses of bacteria to changes in environmental conditions, the uptake of some fermentable sugars, and the intracellular fluxes of carbon intermediates, are regulated by means of reversible protein phosphorylation (for recent reviews, see references 8, 17, and 20). When intact bacteria are labeled in vivo with $^{32}$P, the number of phosphoproteins detected varies considerably among genera. In Escherichia coli, approximately 130 phosphoproteins have been detected by two-dimensional gel electrophoresis (7), whereas at least 15 phosphoproteins were found in Clostridium sp. (1). Most of the phosphoproteins in these and other bacteria are as yet unidentified. More importantly, considerable differences are seen in the patterns and intensities of $^{32}$P-labeled proteins under different growth conditions. For example, the phosphoprotein bands differ in Bradyrhizobium japonicum, in cells growing ex planta and in bacteroids (11), and in Bacillus subtilis, during the sporulation, germination, and outgrowth stages of the growth cycle (12). Variation with growth condition is also seen in the phosphoproteins of the anaerobic sporeformer Clostridium thermohydrosulphuricum (15). Such variation suggests that there are regulatory systems involving protein phosphorylation and dephosphorylation that have not yet been recognized.

In common with other organisms, Clostridium acetobutylicum responds to various stresses by inhibiting the de novo synthesis of most proteins while stimulating the synthesis of specific heat shock or stress proteins (23). In the absence of an applied stress, these organisms exhibit a general synthesis of some stress proteins as they progress from the acidogenic to the solventogenic phase of growth, possibly as a result of the stress imposed by the synthesized solvents (23). Moreover, cultures that are in the solventogenic phase or that are subjected to temperature upshift and butanol stress accumulate diadenosine-5',5''-P',P'-tetraphosphate (Ap$_4$A) and adenosine 5'-P',P'-tetraphospho-5'-guanosine (Ap$_4$G) (2). These nucleotides have been implicated as regulators of stress responses (3, 25).

This study was initiated to investigate whether protein phosphorylation is involved in the stress response in C. acetobutylicum. Intact cells were stressed by temperature upshift or by n-butanol addition, and protein phosphorylation was examined after in vivo labeling with $^{32}$P, or in vitro labeling of cell extracts with [$\gamma$-32P]ATP. The phosphorylation in vivo, but not in vitro, of at least one protein was found to be enhanced in heat-stressed cells. Ap$_4$A, however, did not influence protein phosphorylation in the in vitro system.

MATERIALS AND METHODS

Growth of cells. C. acetobutylicum ATCC 4259 cells were grown under anaerobic conditions at 28°C as previously described (22). The growth phases were identified from the fermentation end products as measured by gas chromatography (2, 22).

Heat and butanol shock. Temperature upshifts from 28 to 45°C and butanol stress were imposed on growing cultures as described previously (23). Heat stress proteins were detected by pulse-labeling 0.5-ml samples of culture with 1 $\mu$M [3H]leucine (40 Ci/mmole) (23). ATP was measured by bioluminometry with the luciferin-luciferase reaction (2).

Gel electrophoresis. Cell samples were prepared for protein electrophoresis by the boiling of samples of cultures as previously described (23). One-dimensional sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (14, 23). The gels were fixed, stained with Coomassie brilliant blue, destained, and, where appropriate, treated with En3Hance (Dupont, NEN Research Products, Boston, Mass). The $^3$H- and $^{32}$P-labeled gels were then exposed to X-ray film (23).

DnaK purification. Two liters of C. acetobutylicum cells was grown to early stationary phase in minimal medium (22), harvested by centrifugation, and suspended in 15 ml of a buffer consisting of 100 mM potassium phosphate (pH 7.0), 100 mM ammonium sulfate, 5 mM mercaptoethanol, 5 mM EDTA, and 10% glycerol. The cells were sonicated with six 15-s pulses at 50 W, and cell extracts were prepared, as described previously (23). The DnaK purification procedure of Zylczik et al. (27) was followed routinely by SDS-PAGE. The purified preparation obtained showed only one Coomassie brilliant blue-stained protein band on gel electrophoresis.

Protein phosphorylation in intact cells. $^{32}$P (0.1 mCi, car-
Protein phosphorylation in developing cells. Cell extracts were prepared from 50-ml cultures grown to the desired phase. One 20-ml sample was centrifuged at 4°C for 7,700 × g for 15 min, while another sample was placed at 45°C for 1 h and then centrifuged. The pellets were suspended in 1 ml of H2O and sonicated six times for 15 s at 50 W.

The reaction mixture contained 30 µl of crude cell extract (175 µg of protein), 10 mM MgCl2, 10 µM Ap5A, 10 µl of [γ-32P]ATP (0.1 mM, 0.8 Ci/µmol), 30 µl of purified DnaK preparation where indicated, and H2O to a total volume of 100 µl. The reaction mixture was incubated for 15 min at 37°C. Trichloroacetic acid-precipitated proteins were analyzed by SDS-PAGE as described above.

RESULTS AND DISCUSSION

Phosphorylation of proteins in intact cells. Growing cultures of C. acetobutylicum were pulsed with 32P, at various times during growth in batch culture. Cell extracts were prepared, and proteins were examined by SDS-PAGE (Fig. 1, even-numbered lanes). The protein bands at approximately 62, 38, and 35-kilodaltons (kDa) were the ones most prominently labeled with 32P, and fainter bands appeared at approximately 96, 88, 83, and 58 kDa (Fig. 1A). The molecular masses of the phosphorylated proteins generally differed from those of C. sphenoides (1) and C. thermohydrosulfacidum (15), the other clostridial species that have been examined. An exception was the prominent protein band at 62 kDa, which may be the same as the 63- to 64-kDa proteins of the other two clostridia. The 83-kDa protein in C. acetobutylicum may be the same as the 82-kDa protein of C. sphenoides.

As the C. acetobutylicum cultures aged from acidogenic to solventogenic phase, there was progressively less incorporation of 32P radioactivity into the protein bands, as seen by comparing their intensities (Fig. 1A, lanes 2, 4, 6, and 8). However, the same bands appeared to be labeled to the same relative extent. Thus, the phosphoprotein profiles did not vary with growth phase, unlike the other clostridial species examined (1, 15).

The focus of this study was whether the stress response in C. acetobutylicum involved regulation by a reversible protein phosphorylation mechanism. It has been clearly shown in a number of bacterial systems, including clostridial species, that growth conditions alter the patterns of phosphorylated proteins (1, 7, 11, 12, 15, 18, 21). However, in E. coli, heat or ethanol stress did not cause changes in the extent of phosphorylation in the 16 heat shock proteins, including DnaK, identified among the 128 phosphoproteins observed in E. coli (7).

Heat stressing the C. acetobutylicum cultures resulted in increased total 32P labeling of proteins at each growth phase tested, as seen by comparing cell extracts after the temperature upshift (odd-numbered lanes) with those of unstressed cells (even-numbered lanes). However, the same protein bands were phosphorylated under the two conditions. In other experiments, the cell extracts were compared by loading each lane with equal radioactive counts instead of equal amounts of protein. Here, there were no differences seen in the phosphorylated protein bands of heat-stressed and unstressed cells (data not shown). Thus, heat stress enhanced the phosphorylation of specific proteins and, interestingly, these did not correspond to stress proteins seen previously for these clostridia in one-dimensional electrophoreograms (23).

To test whether the phosphorylated protein bands included stress proteins, the cultures shown in Fig. 1A were also assayed for de novo synthesis of heat shock proteins by being pulsed with [3H]leucine (Fig. 1B). As reported previously (23), when the culture aged, total de novo protein synthesis decreased, as seen by comparing the incorporation of radiolabeled amino acid in the unstressed cells as they progressed from acid-phase to solvent-phase cells (Fig. 1B, lanes 2, 4, 6, and 8). The synthesis of specific, 3H-labeled heat shock or stress proteins, on the other hand, was stimulated (odd-numbered versus even-numbered lanes). The stress protein bands seen in these extracts were most prominent at 83, 74, 68, and 49 kDa (Fig. 1B; 23). These bands did not correspond to the phosphorylated proteins.
seen in one-dimensional gels of the same cell extracts, with the possible exception of the phosphorylated band at approximately 83 kDa. Interestingly, in this organism, several stress proteins have been detected in two-dimensional, but not in one-dimensional, gel systems, including spots at 62, 49 (2 spots), 36, and 18 (2 spots) kDa (23). The gels were also stained with Coomassie brilliant blue to indicate the relative protein content of the various protein bands (Fig. 1C). None of phosphorylated protein bands were present in large amounts.

Protein phosphorylation by cell extracts. Incubation of cell extracts of *C. acetobutylicum* with [γ-32P]ATP and subsequent SDS-PAGE revealed a number of radioactive protein bands (Fig. 2). The most prominent bands were at 62 and 35 kDa, which were also phosphorylated most extensively in intact cells (Fig. 1). The 38-kDa phosphoprotein band seen in intact cells was absent in the in vitro phosphorylation system, as were the 88- and 83-kDa bands. Some differences in the phospholabeling of proteins in vitro and in vivo have also been reported in other systems (15). Extracts from heat-stressed *C. acetobutylicum* cells showed a phosphorylated band at 96 kDa (Fig. 2, lanes 7 and 8) not seen in unstressed cells (lanes 3 and 4).

Role of DnaK in protein phosphorylation in *C. acetobutylicum*. We were particularly interested in a possible role for the cloridial DnaK, identified previously as a stress protein in *C. acetobutylicum* (24), in protein phosphorylation in stressed cells. DnaK plays a role in turning off the heat shock response in *E. coli* (24). Although the function of DnaK is not completely understood (6), the protein is essential for normal growth (5) and is required for DNA replication (18, 19); DnaK may act as an environmental sensor (6) and may participate in the degradation of abnormal proteins (13). In *E. coli*, DnaK has been shown to possess ATPase activity and autophosphorylating activity (28) and is phosphorylated during growth (18). It also participates in the phosphorylation of other proteins, including two aminoacyl-tRNA synthetases (26). The 5′-nucleotidase activity of DnaK is inhibited by Ap₅A (4), which had been postulated to belong to the group of alarmones, or cellular stress signal nucleotides (3, 25). However, evidence from *E. coli* mutants which overproduce or underproduce the dinucleotide suggests that Ap₅A is not a signal for the induction of heat shock response (9, 16).

The clostridial 74-kDa heat shock protein, which cross-reacts with *E. coli* anti-DnaK antiserum (23), was prepared by previously published procedures (27) so that only one Coomassie brilliant blue-stained band was visible on gels (data not shown). When tested with AMP or ADP as the substrate, the clostridial preparation showed no 5′-nucleotidase activity (data not shown). We next assayed the DnaK preparation for ATPase activity, as a possible reflection of kinase activity in the absence of the protein substrate. The purified DnaK preparation exhibited ATPase activity that was sensitive to Ap₅A when the dinucleotide concentration exceeded that of the substrate, ATP. It is unlikely that the Ap₅A effect is biologically significant, since the effects were observed at Ap₅A concentrations (100 µM) which are considerably higher than that (<0.4 µM) found in the cells (2). Moreover, in intact cells, ATP concentrations are approximately 1,000-fold greater than those of Ap₅A.

In contrast to the enzyme from *E. coli* (28), purified DnaK from *C. acetobutylicum* cells did not show autophosphorylation when tested with [γ-32P]ATP, as there was no phosphorylated band visible at 74 kDa, the molecular mass of clostridial DnaK (23) (data not shown). The DnaK preparation, however, was active as a kinase to specifically phosphorylate a protein of approximately 50 kDa. This protein was present at a low concentration in the DnaK preparation, as it was not seen in Coomassie brilliant blue-stained gels. The 50-kDa protein was present in larger amounts in crude cell extracts, as its phosphorylation was prominent in cell extracts enriched with purified DnaK (Fig. 2, lanes 1 and 2). Moreover, the phosphorylation of the 50-kDa protein was enhanced in extracts from heat-stressed cells (Fig. 2, lanes 5 and 6). Finally, there was no evidence for the involvement of Ap₅A in protein phosphorylation by cell extracts under any condition tested (Fig. 2, odd-numbered lanes versus even-numbered lanes).

Effect of butanol on in vivo protein phosphorylation. We had found previously that stress proteins are synthesized in response to the addition of as little as 0.1% butanol to intact cells (23), although this treatment did not significantly alter the Ap₅A content of the cultures (2). In contrast, with 1.0% butanol addition, Ap₅A in the culture increased significantly. By comparing the effects of these two stresses, it might be possible to distinguish, in intact cells, between Ap₅A-affected protein phosphorylation and a response not involving the dinucleotide. We found that the phosphorylated protein bands of extracts prepared from cells late in the acid phase were not affected by 0.1% butanol addition to the cultures (Fig. 3, lanes 7 and 8 compared with lanes 1 and 2), whereas temperature upshift (lanes 3 and 4) increased protein phosphorylation. Addition of 1% butanol, which is known to permeabilize the cell membrane (10), decreased protein phosphorylation, probably because of the loss of intracellular metabolites (lanes 5 and 6). Again, the evidence...
FIG. 3. Effect of butanol and heat stress on protein phosphorylation in intact C. acetobutylicum cells. $^{32}$P was added to cells late in the acidogenic phase, and various stresses were imposed for 1 h before the cells were harvested for analysis, as described in the text. Lanes: 1 and 2 (duplicates), no treatment; growth at 28°C; 3 and 4, heat-stressed cells, cultures shifted to 45°C; 5 and 6, addition of butanol to 1% (vol/vol); 7 and 8, addition of butanol to 0.1%. The phosphorylated bands visible on the gel are, from the top down, 88, 62, 58, 38, and 35 kDa, with fainter bands at 96 and 83 kDa.

Suggested that ApppA performs no role in imposed stress or growth-related stress in C. acetobutylicum.

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