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Effect of Cobalt on Synthesis and Activation of *Bacillus licheniformis* Alkaline Phosphatase

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The effect of CO^{2+} on the synthesis and activation of *Bacillus licheniformis* MC14 alkaline phosphatase has been shown by the development of a defined minimal salts medium in which this organism produces 35 times more (assayable) alkaline phosphatase than when grown in a low-phosphate complex medium or in the defined medium without cobalt. Stimulation of enzyme activity with cobalt is dependent on a low phosphate concentration in the medium (below 0.075 mM) and continued protein synthesis. Cobalt stimulation resulted in alkaline phosphate production being a major portion of total protein synthesized during late-logarithmic and early-stationary-phase culture growth. Cells cultured in the defined medium minus cobalt, or purified enzyme partially inactivated with a chelating agent, showed a 2.5-fold increase in activity when assayed in the presence of cobalt. Atomic spectral analysis indicated the presence of 3.65 ± 0.45 g-atoms of cobalt associated with each mole of purified active alkaline phosphatase. A biochemical localization as a function of culture age in this medium showed that alkaline phosphatase was associated with the cytoplasmic membrane and was also found as a soluble enzyme in the periplasmic region and secreted into the growth medium.

Previous physicochemical studies of the alkaline phosphatase (APase; orthophosphoric monoester phosphohydratase; E. C. 3.1.3.1) produced by the facultative thermophile *Bacillus licheniformis* MC14 were carried out on cells cultured in a low-phosphate complex medium, Neopeptone (Difco). A minimal salts medium has been developed in which *B. licheniformis* synthesizes 35-fold more APase activity than when cultured in Neopeptone.

APase has been most extensively studied in *Escherichia coli* (21). Synthesis of APase in *E. coli* K-12 is regulated by at least four genes, *phoS, phoT, phoR,* and *phoB* (2, 3, 5, 16, 27). Studies in bacilli indicated that genetic regulation is similar to that of *E. coli* (15). *E. coli* APase is a metalloenzyme containing 4 g-atoms of zinc per mol, which appears to affect both structural and catalytic properties of the enzyme (30). Inactivation by metal depletion has been reversed with the addition of either zinc or cobalt in *E. coli* (13) and *Bacillus subtillis* (29).

Genetic and biochemical studies of the regulation of APase synthesis have revealed that a number of factors influence the synthesis of this enzyme. Although P_i derepression has been considered a primary regulatory mechanism (25) with orthophosphate itself as a corepressor (6, 25), Wilkins (26) demonstrated that, in *E. coli*, certain nucleotide species regulated APase synthesis regardless of the phosphate concentration in the growth medium.

Ghosh and co-workers reported that carbohydrate metabolism and the external phosphate concentration in the growth medium directly affect APase synthesis, whereas the intracellular inorganic and total phosphate pools are unrelated to repression or derepression of APase in *B. subtilis* SB15 and *B. licheniformis* 749/C (8, 12). The observation has been made that variations in growth media (rich or minimal) have a distinct effect on APase production (quantity and isoenzyme type) and location (8, 9, 20, 28).

We report in this paper the effects of phosphate and cobalt on the production and location of APase synthesized in a chemically defined medium by *B. licheniformis* MC14. The cobalt effect, a 35-fold increase in APase activity, was studied with respect to protein synthesis, enzyme activation, and metal content of the APase.

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MATERIALS AND METHODS

Organism and growth conditions. The isolate used in this study was a facultatively thermophilic strain of *B. licheniformis* MC14 (10). The following media and procedures were developed to increase the production of APase. The defined minimal medium (DMM) was composed of: ammonium sulfate, 30.27 mM; sodium citrate, 6.88 mM; ferric chloride, 0.304 mM; manganese sulfate, 0.0028 mM; magnesium sulfate, 1.62 mM; potassium phosphate, 0.4 mM; and fructose, 67.5 mM (pH 6.8). The defined medium (DM)

used for increased cell growth was composed of the same component concentrations found in DMM, with the following additions: ferric chloride, 3.04 mM; Trizma base, 50 mM; and fructose, 134 mM (pH 7.12). For maximum production of APase, $CoCl_2$ (0.1 mM) was added to DM medium, thus designated $DMCo^{2+}$. DM agar stock plates were inoculated from tryptone agar plates, incubated for 18 h at 55°C, and stored at 4°C. DMM plates were inoculated from stock plates and incubated (12 h) at 55°C to serve as an inoculum for a DM liquid culture. Culture conditions were as follows: flasks (1,000 ml, baffle bottomed) containing 400 ml of DM were inoculated to an initial absorbance of 0.17 at 540 nm (1-cm cuvette). The culture was grown for 12 h at 50°C in a New Brunswick environmental shaker at 350 rpm to an absorbance at 540 nm of 1.8 to 2.0. For APase production, cells from DM cultures were used to inoculate flasks containing 400 ml of $DMCo^{2+}$ medium. Culture conditions were identical to those described for DM medium.

Assays. Alkaline phosphatase was assayed as described by Hulett and Campbell (11) unless otherwise indicated. One unit of alkaline phosphatase is defined as the amount of enzyme which liberates 1 μmol of *p*-nitrophenol per min under the defined conditions. Aldolase was assayed by the method of Rutter (22). Glycerol dehydrogenase was assayed as described by Strickland and Miller (23).

Protein concentrations were determined by the modified biuret method of Burgi et al. (4).

Inorganic phosphate concentrations were determined by the method of Ames (1).

Effect of chloramphenicol on cobalt-stimulated APase activity. Four 1,000-ml flasks each containing 400 ml of DM were inoculated to an absorbance of 0.2 at 540 nm. After 5 h of growth (absorbance at 540 nm of 1.55 to 1.65, and APase activity between 0.4 and 0.6 U/ml), the following additions were made to individual flasks: flask 1, no addition; flask 2, $CoCl_2$ (0.1 mM); flask 3, $CoCl_2$ (0.1 mM) and 0.25 ml of methanol; flask 4, $CoCl_2$ (0.1 mM) and 80 mg of chloramphenicol, dissolved in 0.25 ml of methanol. The cell growth and enzyme activity were assayed 0, 5, 15, 25, 40, 60, 90, and 165 min after the additions.

Biochemical localization of APase in $DMCo^{2+}$ as a function of culture age. Cells were inoculated into a $DMCo^{2+}$ medium, as described above, to an initial absorbance of approximately 0.170 at 540 nm. The growth and enzyme activity were monitored until the culture reached an absorbance of 1.2 at 540 nm.

Protoplast formation and fractionation were performed as described by Glynn et al. (9), except that 20% glycerol, 0.03 M $MgSO_4$, 2 mg of lysozyme per ml, and a 25°C incubating temperature replaced 0.8 M sucrose, 0.05 M $MgSO_4$, 15 mg of lysozyme per ml, and a 37°C incubating temperature, respectively, in the protoplast forming solution. (Twenty percent glycerol is necessary to stabilize the otherwise unstable periplasmic APase.) After 90 to 95% protoplast formation, as determined by phase-contrast microscopy, the $MgSO_4$ concentration was raised to 0.05 M, and the suspension was centrifuged at 5,000 × *g* for 30 min. Further fractionation procedures were identical to those described by Glynn et al. (9).

Analytical determination of metal ions. Metalloalkaline phosphatases were prepared by adding 10^{-3} M metal concentration of a specific metal ion contained in 0.01 M Tris-acetate (pH 7.3) to an enzyme preparation which had been dialyzed against the same buffer until the enzymatic activity was zero. After 24 h in the presence of the added metal, the samples were dialyzed to equilibrium against 400 volumes of 0.01 M Tris-acetate (pH 7.3). Both the dialysate and the protein sample were analyzed for metal content.

Analytical determinations of metal ions were carried out by flameless atomic absorption spectroscopy using a carbon rod atomizer.

RESULTS

Effect of cobalt on APase production. When *B. licheniformis* MC14 was grown in 1% Neopeptone, a maximum activity of 0.2 U/ml was obtained (9). Figure 1 shows the growth curve and enzyme production of cells grown in DM minus cobalt (Fig. 1A), in DM to which 0.1 mM cobalt was added during the late exponential phase (Fig.1B), and in DM containing 0.1 mM cobalt at zero time ($DMCo^{2+}$) (Fig. 1C). The growth curves of all three experiments were similar. However, the enzyme activity was increased from 0.4 U/ml in experiment A to 4.75 U/ml in B and 7.0 U/ml in C. Experiments to determine the optimal cobalt concentration showed that 0.1 mM cobalt stimulated the greatest production of APase activity in a growing culture. $DMCo^{2+}$ is a phosphate-limiting medium which does not cause sporulation in the organism even when the culture is allowed to proceed into death phase.

Effect of phosphate concentration on cobalt-stimulated APase production. When cells were grown in DM containing cobalt (0.1 mM), the phosphate concentration decreased from 0.4 mM to approximately 0.075 mM before APase synthesis occurred (Fig. 2). When cells were grown in DM without cobalt, APase production occurred after the phosphate concentration decreased below 0.1 mM, but at a much slower rate. Figure 3A shows the growth curve, the decrease in phosphate during growth, and APase production in a 1,000-ml culture. When the phosphate concentration reached 0.05 mM, the culture was divided into two 500-ml cultures. Cobalt (0.1 mM) was added to one flask but not to the other. The APase activity increased in the flasks with cobalt and without cobalt from 0.30 U/ml to 7.5 and 0.5 U/ml, respectively, within 4.5 h. When a similar experiment (Fig. 3B) was carried out (except that, in addition to cobalt, 0.3 mM phosphate was added to each 500-ml culture), APase synthesis in both flasks was similar (0.4 and 0.6 U/ml) within the next 4.5 h.

From these data it is clear that cobalt is necessary for maximal stimulation of APase activ-

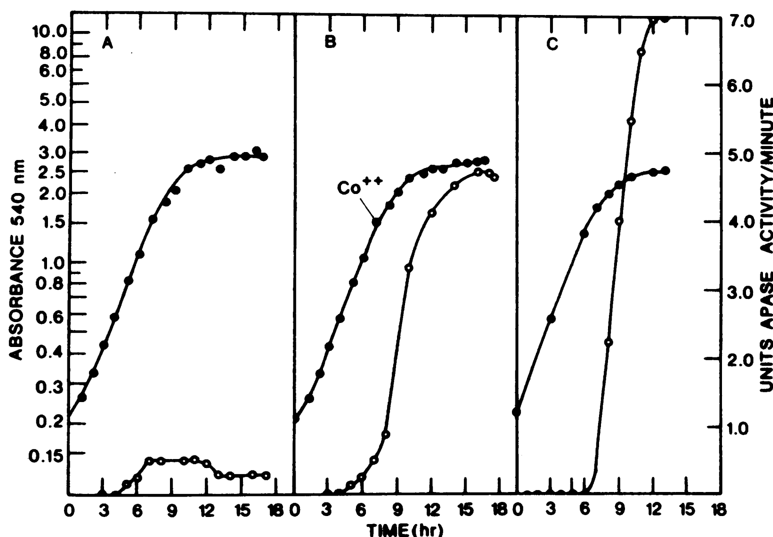

FIG. 1. *Effect of cobalt on growth and enzyme production of B. licheniformis in low-phosphate DM. (A) Growth and enzyme production in DM without cobalt. (B) Growth and enzyme production in DM to which 0.1 mM $CoCl_2$ was added after 7 h. (C) Growth and enzyme production in $DMCo^{2+}$ medium. Symbols:* (●) *growth;* (O) *enzyme production.*

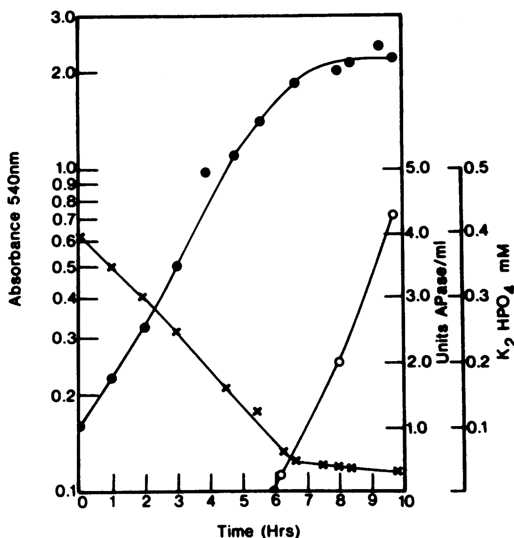

FIG. 2. *Correlation of the phosphate concentration and the initiation of alkaline phosphatase production on $DMCo^{2+}$. Symbols:* (●) *growth;* (O) *enzyme production;* (X) *phosphate concentration.*

ity, but this stimulation is inhibited by phosphate concentration in the medium of over 0.075 mM.

The effects of other metals on APase activity were determined when these metals were added to the defined medium in concentrations of 0.1 mM or 0.1 mM above that already in the medium. Manganese stimulation was 25% and magnesium, sodium, or iron stimulation was 15% of the activation observed with cobalt. Zn^{2+} did not stimulate APase production. None of these metals had any additional stimulatory effect on APase activity, nor any effect on growth when added to $DMCo^{2+}$.

Synthesis of APase in relation to total protein synthesized. Samples were taken from a $DMCo^{2+}$ culture hourly between 5 and 9 h. During this time the activity increased from 0.9 to 7.0 U/ml. The units of APase per milliliter and total protein (milligrams per milliliter) were determined. Using the highest specific activity for purified APase from *B. licheniformis* MC14 (10), the milligrams of APase per ml of each sample was calculated (Table 1); approximately 56% of the total protein synthesized was APase.

Biochemical localization. Total APase activity was determined by assaying 25-ml samples harvested at various stages of growth. Enzyme activity was monitored in the DM medium from which the cells were harvested, in the soluble fraction released upon protoplast formation, in the membrane fraction, and in the cytosol fraction. Figure 4 shows the distribution of enzyme activity found in these four locations. Significant amounts of APase activity were not apparent until h 5 of growth. During the first 1.5 h of APase synthesis, 63% of the total APase activity appeared in the periplasmic space, while the concentration of active membrane-associated enzyme reached a maximum of 32%. The medium contained less than 4% of the enzyme

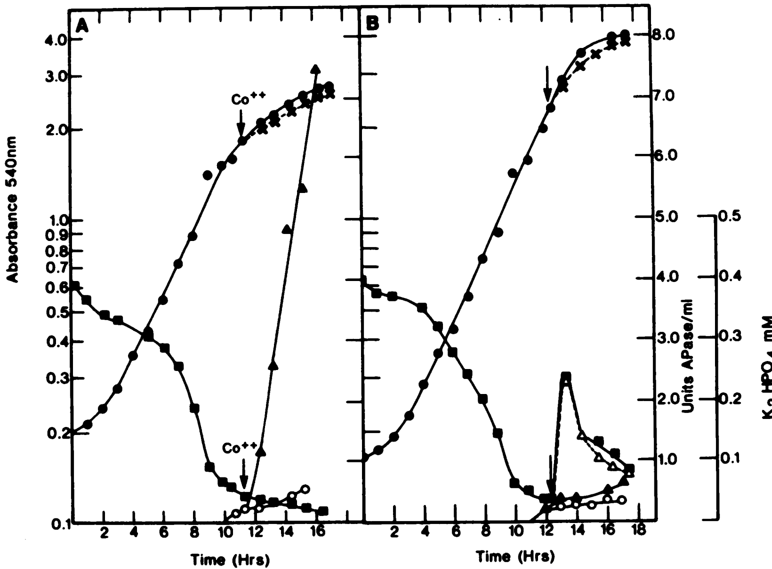

FIG. 3. *Effect of phosphate concentrations on cobalt-stimulated APase activity. (A) Comparison of phosphate utilization and APase production in DM and DMCo^{2+}. Symbols:* (●) *growth;* (■) *phosphate concentration in the medium;* (×) *growth in the flask that received 0.1 mM cobalt;* (○) *enzyme production in flask without cobalt;* (▲) *enzyme production in the flask with 0.1 mM cobalt. (B) Conditions of growth were identical to the cultures in (A) except both flasks received phosphate (0.3 mM phosphate) when cobalt was introduced into one of the two flasks. Symbols:* (●) *growth,* (×) *growth in flask that received cobalt (0.1 mM) and phosphate (0.3 mM);* (■) *phosphate concentration in the medium;* (△) *phosphate concentration in the flask that received cobalt (0.1 mM) and phosphate (0.3 mM);* (▲) *enzyme activity in the flask that received cobalt (0.1 mM) and phosphate (0.3 mM);* (○) *enzyme production in the flask that received phosphate (0.3 mM) but no cobalt.*

TABLE 1. *Total protein synthesized during maximal APase production*[a]

Sample time (h)	Protein: Content (mg/ml)	Protein: Δ Protein content (9 h − 5 h)	Activity: APase activity (U/ml)	Activity: Δ Activity (9 h − 5 h)
9	0.1719		7.11	
5	0.1221	0.0498	0.78	6.33

[a] Specific activity of purified APase = 228 U/mg. Therefore 6.33 U = 0.028 mg of APase, and (0.028 mg of APase)/(0.0498 total protein) × 100 = 56% of total protein synthesized.

activity at this time, whereas the soluble cytosol fraction showed no enzyme activity, nor was any activity found in the cytosol for the entire growth period. During the next 105 min the concentrations of membrane-bound and soluble periplasmic space enzyme activities plateaued at 36 and 26 U of activity per 25-ml sample, respectively. A gradual rise in soluble APase in the medium was observed. Glycerol dehydrogenase and aldolase (soluble cytosol enzymes) were not released into the growth medium during the entire localization. During stationary phase, the concentration of enzyme on the membrane in-

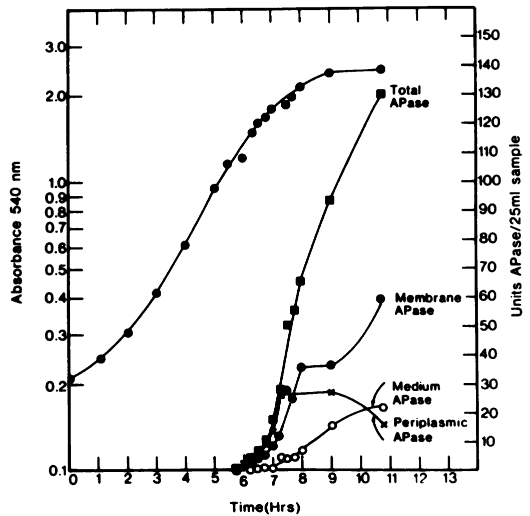

FIG. 4. *Distribution of APase as a function of culture age. Growth curve* (●). *Total APase activity in the 25-ml sample at harvest* (■); *in the soluble fractions of culture medium* (○); *released upon protoplast formation* (×) *after 1 h of 100,000 × g centrifugation; and associated with cytoplasmic membrane* (⊗). *No soluble alkaline phosphatase was released upon protoplast lysis.*

creased significantly to almost 60 U per 25-ml sample (a 100% increase), whereas the periplasmic enzyme showed a decline in enzyme activity of 75%.

Effect of chloramphenicol on cobalt-stimulated APase activity. Studies were conducted to determine whether the synthesis of protein is necessary for stimulation of APase activity by cobalt, or if the rapid stimulation is the activation of preformed apoenzyme or enzyme precursor. Figure 5 shows the growth curves and the enzyme activities in the experimental flask and a $DMCo^{2+}$ control flask. Control flasks containing $CoCl_2$ plus methanol or DM (no cobalt) were also grown (data not shown). Before the additions made after 5 h of growth, the growth curves and enzyme activity of the culturres in all the flasks were quite comparable. The culture that received chloramphenicol stopped growing immediately; enzyme activity in this flask increased from 0.35 to 0.6 U/ml directly after the addition of chloramphenicol and cobalt, but failed to increase further. The control cultures containing $CoCl_2$ or $CoCl_2$ plus methanol grew normally and produced similar amounts of enzyme. The control which contained no cobalt showed growth and enzyme production typical for these conditions (see Fig. 1A).

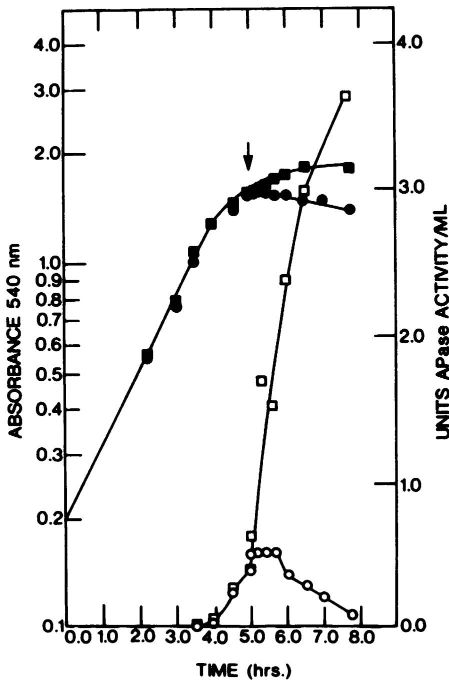

FIG. 5. *Effect of chloramphenicol inhibition on protein synthesis and cobalt-stimulated APase activity. The solid symbols represent growth, and the open symbols represent enzyme production. Symbols:* (■, □) *Control flask which received 0.1 mM cobalt after 5 h of growth;* (●, ○) *experimental flask which received chloramphenicol (final concentration in the flask, 200 μg/ml) dissolved in 0.25 ml of methanol and cobalt (0.1 mM) after 5 h of growth.*

Effect of cobalt stimulation on whole culture activity. APase activity of DM and $DMCo^{2+}$ cultures was assayed with and without 0.1 mM $CoCl_2$ in the assay mixture. Cobalt in the sample mixture caused less than a 1% stimulation when $DMCo^{2+}$-grown cells were assayed. An increase in APase activity of as much as 2.5-fold was observed (never greater than 0.65 U/ml) when DM-grown cells were assayed in the presence of cobalt.

Effect of ions on APase activity. Samples of purified medium APase (purification to be reported elsewhere) were dialyzed for 18 h against 1,000 volumes of 0.01 M Tris-acetate (pH 7.3) or the same buffer containing 5 mM EDTA. EDTA was removed from the EDTA-treated samples by dialysis for 24 h against 1,000 volumes of 0.01 M Tris-acetate (pH 7.3). The APase showed a 75% and 100% loss in activity when dialyzed against Tris and Tris containing 5 mM EDTA, respectively. Partial activity was restored to samples dialyzed against Tris when assayed in the presense of several metals, with Co^{2+} and Mg^{2+} being the most effective (Table 2). The EDTA-treated enzyme could not be reactivated by inclusion of metals (10^{-3} M) in the assay or by incubation with excess metal (5×10^{-3} M) for 4 h before the enzyme was assayed.

Metal ion content of APase. Purified APase which had been dialyzed exhaustively against 0.1 M Tris-acetate (pH 7.3) until all the enzymatic activity was removed contained 1.96 g-atoms of Co^{2+} and 0.45 g-atoms of Zn^{2+} per mol of APase. The metal content of APase which had been dialyzed against the same buffer containing 5 mM EDTA was too low for detection with the quantities of protein available. When metalloenzymes were prepared from Tris-dialyzed inactive APase, cobalt restored 60 to 70% of the activity and the enzyme contained 3.65 $\pm$ 0.45 g-atoms of Co^{2+} per mol. A similar experiment with Zn^{2+} restored only about 10% of the activity, and the APase contained about 0.25 g-atoms of Zn^{2+} per mol.

DISCUSSION

Hydrean et al. have reported the development of a chemically defined medium in which *B. licheniformis* 749/C produced 0.22 U of APase per mg of cells (units defined as micromoles of *p*-nitrophenol produced per minute) (12). This was comparable to the APase production of *B.*

TABLE 2. *Effects of ions on the relative enzyme activity of APase*

Ion	Concn in assay (mM)	Relative activity after dialysis in Tris
None		1.00
Mg^{2+}	0.1	1.23
	1.0	1.94
	5.0	2.19
Co^{2+}	0.1	2.30
	1.0	2.27
	5.0	2.41
Mn^{2+}	0.1	0.31
	1.0	0.29
	5.0	0.30
Zn^{2+}	0.1	1.00
	1.0	1.35
	5.0	1.36
Ca^{2+}	0.1	1.14
	1.0	1.15
Cu^{2+}	0.1	1.10
	1.0	0.98
Co^{2+} + Mn^{2+}	1 + 1	2.68
Co^{2+} + Mg^{2+}	1 + 1	2.50

licheniformis MC14 when cultured in 1% Neopeptone (0.2 U of APase per mg of cells) (9). We here the factors which affect the synthesis of APase by *B. licheniformis* MC14 in our defined medium, which supports twice the cell density of 1% Neopeptone cultures and a 35-fold increase in APase production (3.5 U of APase per mg of cells).

The cobalt-stimulated APase activity was decreased in the presence of additional phosphate from 7.5 to 0.6 U/ml, even though the culture density doubled with this addition. The APase activity in the medium minus cobalt, with or without added phosphate, showed very little difference in APase production (0.4 and 0.5 U of APase per ml, respectively) because the APase is not totally repressed in the presence of higher levels of phosphate (14) and the culture density increases significantly in the presence of additional phosphate.

The increased activity may be the result of both enhancement of the enzymatic activity by cobalt and stimulation of synthesis of the enzyme.

The enzyme can be inactivated by exhaustive dialysis against 0.01 M Tris (pH 7.3) or shorter dialysis against the same buffer containing EDTA. (The "Good" buffers have recently been shown to be metal chelating agents [17].) The APase, dialyzed against Tris, contained approximately 2 atoms of cobalt per molecule and had less than 1% of the original activity. Cobalt restored 60 to 70% of the activity, and the metal content increased to 3.65 ± 0.45 g-atoms of Co^{2+} per mol. When enzyme was dialyzed in buffer containing 5 mM EDTA, activity was reduced to zero, no associated metal could be detected, and we were unable to restore any activity by addition of metal to the reaction or by extensive preincubation in the presence of high concentrations of metal. These data indicate that there are probably four Co^{2+} atoms associated with the active APase; two are relatively labile atoms essential for activity, and the two more tightly bound atoms maintain the conformational stability of the enzyme.

Similar studies in *B. subtilis* (29) and *E. coli* (18, 24) have implicated as few as 2 and as many as 6 g-atoms of metal per mol of APase. Studies by Szajn and Csopak (24) indicated that the titration of the apoenzyme of *E. coli* with Zn^{2+} leads to a linear increase in activity, with maximal activity being obtained at four Zn^{2+} ions per dimer. These data concur with previous studies which suggested that one pair of Zn^{2+} atoms appear to play a predominantly structural role, while the second pair is involved primarily in catalysis (19). All of the metal ions which bind to *E. coli* APase (Co^{2+}, Ni^{2+}, Cu^{2+}, Mn^{2+}, Fe^{2+}, or Hg^{2+}) are exchange labile. However, only Co^{2+} and Zn^{2+} are capable of restoring catalytic activity of *E. coli* APase (30) or *B. subtilis* APase (29). *B. subtilis* APase apoenzyme reactivation after metal extraction was much more difficult than reactivation of *E. coli* APase. Fifty percent of the initial activity was restored with Zn^{2+} (2.48 ± 0.5 g-atoms of zinc per dimer), whereas Co^{2+} restored 100% of the initial activity.

Protein synthesis inhibition studies with chloramphenicol indicated that a majority of the Co^{2+}-stimulated APase activity could be inhibited with the termination of protein synthesis (an increase of approximately 25-fold was prevented). However, the simultaneous addition of chloramphenicol and cobalt (10^{-4} M) to a DM culture allowed for an immediate twofold increase in APase activity (from 0.3 U of APase activity per ml to 0.6 U of activity). This can be explained as activation of preformed enzyme, since whole-culture assays of DM-grown cells assayed with or without Co^{2+} in the reaction mixture resulted in a 2.5-fold activity increase in the presence of Co^{2+}.

The fact that the majority of APase stimulation observed when Co^{2+} is added to DM cultures is stopped by inhibition of protein synthesis can be explained in one of two ways. Either

Co^{2+} influences some component of the regulatory mechanism for APase synthesis, or protein synthesis is necessary because only the nascent APase peptide can associate with Co^{2+} to form a subunit with the correct conformation to allow functional activity. (This would explain our inability to renature apoenzyme.) The latter suggestion would assume that protein synthesis of APase occurs at the same rate in DM and $DMCo^{2+}$, but the absence of an adequate concentration of cobalt results in the production of functionally inactive APase which would be susceptible to proteolytic digestion. One must hypothesize that without added cobalt either (i) there is a high turnover rate of such a nonfunctional APase, (ii) synthesis of APase does not occur, or (iii) nonfunctional APase does not copurify with the active dimer since the specific activity of purified APase from cells grown in media with or without added cobalt is essentially the same (Hulett, unpublished data).

We calculated that over 50% of the protein synthesized during late-exponential and early-stationary phases of growth is APase. Although it is unlikely that APase in vivo has the same specific activity as purified APase, if APase in vivo were twice as active as isolated APase, the amount of protein synthesis involved in APase production would still be significant (over 25%).

Not only was there a substantial increase in the amount of active APase produced when cells were grown in $DMCo^{2+}$, but a markedly different distribution of the enzyme as a function of culture age was observed. The decline in periplasmic enzyme may not reflect the in vivo situation, since this unstable APase (9) may (during fractionation) be susceptible to proteolytic activity. The most significant difference observed was the secretion of enzyme into the culture medium. Neither of the previous studies (9, 14) indicated activity in the culture medium, nor did total culture activity rise above 0.2 U/ml.

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