Phosphorylation of Ribosomal Protein L18 Is Required for Its Folding and Binding to 5S rRNA

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ABSTRACT: Ribosomal protein L18 from *Bacillus stearothermophilus* (bL18) includes a previously unreported phosphoserine residue. The folded conformation of the protein is stabilized by the dianionic form of the phosphate group of that residue. In the absence of Mg^{2+}, the pK_a of the phosphate group is so high that the protein is not fully folded at pH 7. In the presence of Mg^{2+}, its pK_a drops significantly, and consequently the native conformation of bL18 becomes stable at pH 7 and the protein is able to bind to 5S rRNA. Dephosphorylated bL18 does not bind to 5S rRNA at neutral pH.

5S rRNA is a conserved component of the large ribosomal subunit that is thought to enhance protein synthesis by stabilizing ribosome structure (*1, 2*). Three proteins bind to it: L5, L18, and L25 (*3*). Incorporation of 5S rRNA into the large subunit depends on their presence and is one of the last steps in ribosome assembly (*4*). L18 binds the most tightly of the three, and its binding stimulates the interaction of L5 with 5S rRNA (*5*). L18 from *E. coli* retains its 5S rRNA binding capacity after the removal of its first 17 residues, but the cleaved protein no longer stimulates the binding of L5 (*6*). Alignment of eubacterial L18 sequences reveals that the majority of its conserved residues are found in its C-terminal region, consistent with that part of the protein being important for RNA binding (Figure 1) (*7*).

In the early stages of an ongoing project to determine the solution structure of L18 from *Bacillus stearothermophilus* (bL18), we were led to examine the effects of pH and divalent cations on the structure of bL18 by difficulties encountered in preparing samples that have a well-defined conformation. For an intracellular protein, the conformation of bL18 is surprisingly sensitive to pH around neutrality, and its response is strongly affected by Mg^{2+}. Both effects appear to be due to the presence of a previously unreported phosphoserine residue in bL18, which is essential for its binding to 5S rRNA at physiological pH. These observations hint at the possibility that ribosomal protein phosphorylation may play a role in regulating protein synthesis or ribosome assembly in bacteria.

MATERIALS AND METHODS

Isolation of L18. Ribosomal protein L18 from *Bacillus stearothermophilus* (bL18) was isolated essentially as described by Ramakrishnan and Gerchman (*8*). In brief, *E. coli* BL21(DE3), carrying a kanamycin-stabilized plasmid with the gene for bL18 downstream of a T7 promoter, was grown either in Luria broth or in a minimal medium containing $(^{15}NH_4)_2SO_4$ (4 g/L) as the nitrogen source. Overexpression was induced with isopropylthiogalactoside when the optical density (OD_{600}) of the cells was 0.6−0.8. The cells were harvested 3 h later, and disrupted using a French press. The resulting lysate was taken up in 0.7 M NaCl, 20 mM PO_4, pH 7, the debris spun down, and the pellet reextracted with the same buffer. The supernatant was diluted to 0.3 M NaCl, 20 mM PO_4, pH 7, loaded onto an S-Sepharose column, and eluted from the column with a salt gradient running from 0.3 to 1.2 M NaCl in 20 mM PO_4, pH 7. Fractions containing bL18 were identified by gel electrophoresis, pooled, dialyzed into 0.3 M NaCl, 20 mM PO_4, pH 7, and repurified on an S-Sepharose column under the same conditions. The identity of the final product was checked by N-terminal sequencing. Ribosomal protein L18 from *E. coli* (EL18) was isolated from intact ribosomes as described previously (*9*).

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                                                        40
mitkvdrnav rkkrharirk kifgtterpR LsVfrsnkhi

                                                        80
yaQiiddtks ativsaStld kefgldstnn teaakkvgel

                                                       120
vAkralekgi kqvvFDrggy lYHGrvkalA daAREaGLef
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FIGURE 1: Sequence of bL18. The sequence of the protein is shown with residues conserved in eubacterial L18s indicated in boldface capitals (*7*).

Circular Dichroism. Circular dichroism (CD) spectra were measured using an Aviv model 60 DS spectropolarimeter, equipped with a temperature controller. The spectra shown below are the average of four scans recorded with a bandwidth of 1.5 nm, a stepsize of 0.5 nm, and a time constant of 4 s. Spectra were recorded from 250 to 200 nm using a cuvette with a 0.2 cm path length, and from 300 to 250 nm using a 1.0 cm cuvette. Following base line correction, the CD signal (in millidegrees) was converted to mean residue ellipticity. Secondary structure was estimated

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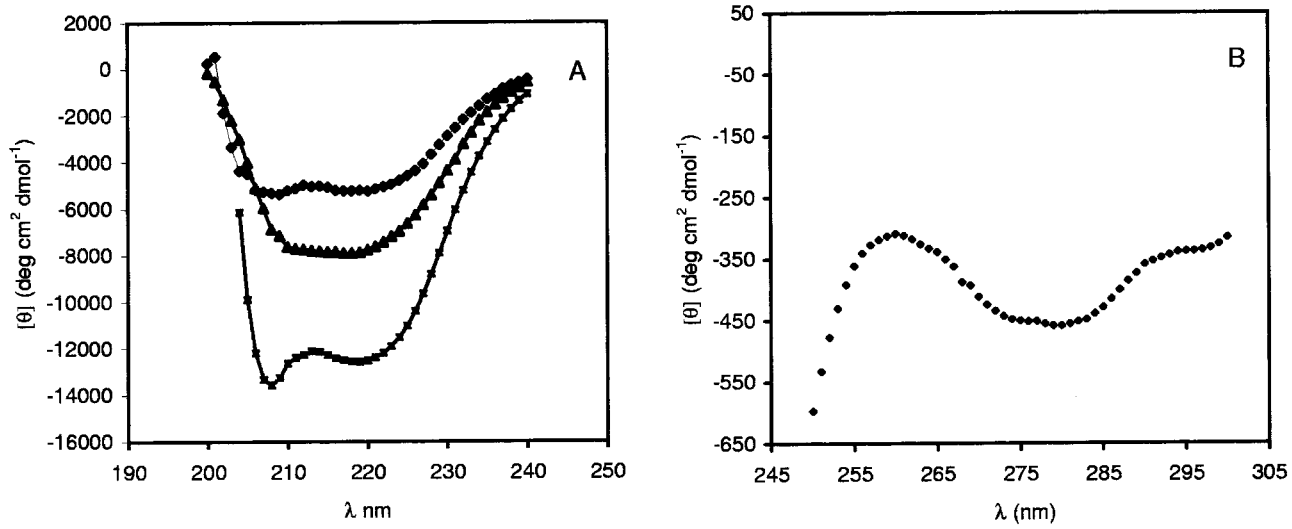

FIGURE 2: Influence of pH on the conformation of bL18 in 0.2 M NaCl, 20 mM PO_4 at 25 °C. (A) Far-UV CD of bL18 at pH 8 (■), pH 7 (▲), and pH 6.5 (◆). (B) Near-UV CD spectrum of bL18 at pH 8 (see Materials and Methods).

from spectra using the curve-fitting algorithm of Fasman (*10*).

NMR Spectroscopy. NMR spectra were recorded on a Varian Unity 500 MHz spectrometer, using either a triple-resonance HCN probe with triple axis gradients or a triple-resonance HCP probe with *Z*-axis gradients. Proton chemical shifts were referenced to a 2,2-dimethyl-2-silopentane-5-sulfonic acid internal standard, and phosphorus chemical shifts were referenced to an external H_3PO_4 standard (85%). $^1H-^{15}N$ HSQC spectra were recorded using a sensitivity-enhanced, pulsed-field gradient HSQC experiment (*11*) with sweep widths of 2000 and 8000 Hz in the indirect (^{15}N) and acquisition (^1H) dimensions, respectively, and with 128 t_1 increments. A total of 16 scans were acquired per t_1 point. ^{31}P NMR spectra were recorded at 202.36 MHz using 30° pulses with a delay time of 1 s and a sweep width of 5000 Hz. For pH titrations, the protein was dialyzed into a buffer solution of the pH required, either in the presence or in the absence of 8 M urea. The buffer used for pHs between 7.0 and 8.0 was 0.2 M NaCl, 20 mM Tris, and for pHs between 4.5 and 7.0, 0.2 M NaCl, 20 mM cacodylate. ^{31}P NMR spectra were measured at 35 °C using ^1H decoupling for the ^{31}P titration data.

Dephosphorylation of L18. The base-labile phosphate esters in L18 preparations were detected by incubating aliquots of L18 in 1 M NaOH for 1 h at 37 °C, and then testing for the presence of inorganic phosphate (*12*). The phosphate was also released using acid phosphatase (from potato). An aliquot of 250 μL of L18, containing 20−30 nmol of protein, was incubated with acid phosphatase (4 mg = 20 units) in citrate buffer (0.2 M NaCl, 0.1 M citrate, pH 4.8) at 37 °C for 4 h, and another 250 μL was incubated in the same buffer in the absence of the enzyme. Aliquots of 62.5 μL were taken from both mixtures at several times, and ice-cold acetone (50%) was added to precipitate the protein. Supernatants were assayed for inorganic phosphate. Pelleted protein was dissolved in 0.1 M KCl, 5 mM $MgCl_2$, 50 mM Tris−borate, pH 7.6, concentrated, and assayed for 5S rRNA binding activity. L18 was treated with alkaline phosphatase the same way except that a buffer containing 0.2 M NaCl, 0.1 mM EDTA, 50 mM Tris, pH 8.5, was used instead of citrate buffer.

Assays. Inorganic phosphate was measured using Ames's colorimetric assay (*13*). One hundred microliters of supernatant, 200 μL of H_2O, and 700 μL of Ames reagent (1 mL of 10% ascorbic acid and 6 mL of 0.42% ammonium molybdate in 1 N H_2SO_4) were incubated for 1 h at 37 °C. The absorbance at 820 nm was measured at room temperature, and $OD_{820\ nm}$ readings were converted into concentrations using a calibration curve obtained with phosphate solutions of known concentration. L18/5S rRNA binding was measured using the gel-shift assay described previously (*14*).

RESULTS

Dependence of bL18 Structure on pH. NMR spectroscopists prefer to study proteins at acid pHs because amide proton resonances are easier to detect at those pHs than they are at neutrality due to a reduced rate of exchange with solvent. However, in typical NMR buffers, e.g., 0.2 M NaCl, 20 mM phosphate, bL18 precipitates below pH 7 when its concentration is in the millimolar range, and rather than search for satisfactory buffer conditions by NMR, which would have cost a lot of protein, we examined bL18's response to ionic conditions by CD spectroscopy, which is more economical.

Figure 2A shows the far-UV region of the CD spectrum of bL18 in 20 mM phosphate buffer at three pH values. This portion of a protein's CD spectrum is strongly influenced by secondary structure, and the bL18 CD curves shown have distinctive negative ellipticities at 208 and 222 nm characteristic of the presence of α-helical structure. Using Fasman's algorithm (*10*), the helix content of the protein was estimated to be about 11% at pH ≤6.5, 18% at pH 7, and 32% at pH 8. [Based on its sequence, the program PHDsec suggests that the helix content of the native form of bL18 should be ∼46% (*15*−*17*).]

The near-UV CD spectrum of a protein reflects the conformation of its aromatic residues, and reports on tertiary structure. In denatured proteins, the near-UV CD spectrum is almost identically zero, as was the case here at pH ≤7.0 (data not shown). At pH 8, however, the three tyrosine and five phenylalanine residues of bL18 make an overall negative contribution to its near-UV CD spectrum (Figure 2B),

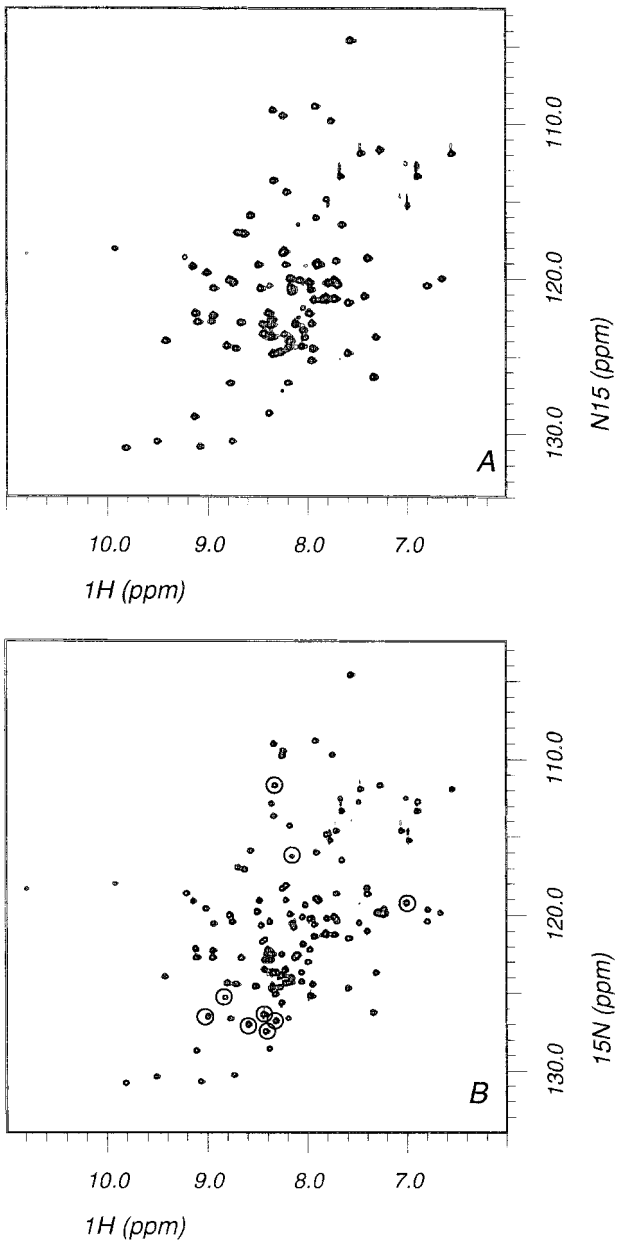

FIGURE 3: Amide region of the $^{15}N-^{1}H$ HSQC NMR spectrum of bL18 at 25 °C. (A) Spectrum in 0.2 M NaCl, 20 mM PO_4, pH 8. (B) Spectrum in 0.2 M NaCl, 5 mM $MgCl_2$, 20 mM PO_4, pH 6. Of the 27 resonances observed at pH 6 that are not visible in the pH 8 spectrum, 9 are circled to aid their identification.

indicative of the presence of tertiary structure. In addition, under the same conditions, the $^{15}N-^{1}H$ HSQC spectrum of bL18 contains 99 cross-peaks (Figure 3A), which is many more than at lower pHs (data not shown). (If all the amino acids of bL18 were represented, a perfectly resolved $^{15}N-^{1}H$ HSQC spectrum of bL18 would contain 118 backbone amide cross-peaks plus 6 pairs of cross-peaks for the side chain amides of its asparagines and glutamines.) Taken together, these observations suggest that at pH 8.0, bL18 adopts a folded conformation, but that at lower pH it does not.

The Folded Conformation of bL18 Is Stabilized by Mg^{2+}. As Figure 4 shows, the ellipticity of the CD spectrum of bL18 increases significantly at pH 6 when its buffer includes 5 mM Mg^{2+}, and curve-fitting calculations indicate that the α-helix content is 35% under these conditions, about what

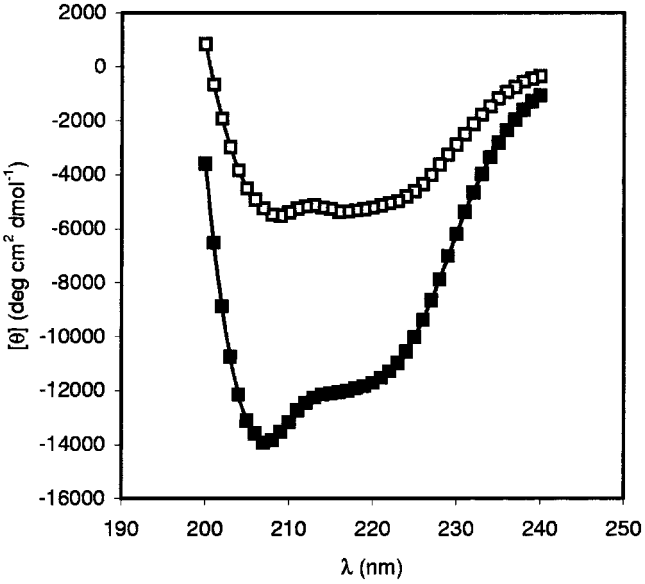

FIGURE 4: Effect of Mg^{2+} on bL18 conformation. Far-UV CD of bL18 at 25 °C in 0.2 M NaCl, 20 mM PO_4, pH 6, in the presence (■) or absence (□) of 5 mM $MgCl_2$.

it is at pH 8.0 in the absence of Mg^{2+}. Also, the near-UV CD spectrum of bL18 at pH 6 in the presence of Mg^{2+} is the same as its near-UV CD at pH 8 in the absence of Mg^{2+}, consistent with the conformation of the protein being the same under both conditions (data not shown). Finally, all of the cross-peaks seen the $^{15}N-^{1}H$ HSQC spectrum of bL18 at pH 8 (Figure 3A) can be found in its pH 6/$MgCl_2$ $^{15}N-^{1}H$ HSQC spectrum (Figure 3B) at almost the same chemical shifts, which is strong evidence that the conformation of bL18 at pH 8 in the absence of Mg^{2+} is indeed the same as it is in the presence of Mg^{2+} at pH 6. The primary difference between the pH 8 spectrum and the pH 6/$MgCl_2$ spectrum is that the latter contains 27 new cross-peaks, some of which are circled in Figure 3B, and several of the weak cross-peaks in the pH 8 spectrum are much stronger in the pH 6/Mg^{2+} spectrum. Both effects reflect the reduction in the rate of exchange between NH groups and solvent protons that results from the difference in pH. Other divalent metal ions, such as Ca^{2+} and Zn^{2+}, do not have this conformation-stabilizing effect, which indicates that the interaction of bL18 with Mg^{2+} is specific.

bL18 Contains a Phosphoserine Residue. All of the observations described above would be explained if bL18 contained phosphorylated residues, and assays for alkaline-labile organic phosphate run on our bL18 samples confirmed that hypothesis. In a typical experiment, 7.1 nmol of bL18 released 6.5 nmol of P_i (see Materials and Methods), indicating the presence of a single phosphorylated residue. To rule out the possibility that the phosphate detected might not be covalently bound, ^{31}P NMR spectra of bL18 were recorded after prolonged dialysis under both native and denaturing conditions. As Figure 5A shows, in urea, the ^{31}P NMR spectrum of L18 includes a triplet resonance at 5.56 ppm, typical of a phosphoserine residue (*18*), that resists prolonged dialysis. The resonance is a triplet because the ^{31}P of the phosphate it represents is coupled to the two β-protons of the serine to which it is esterified, and the $^{3}J_{POCH}$ coupling observed (20 Hz) indicates that one β-proton is gauche and the other is trans with respect to the phosphorus

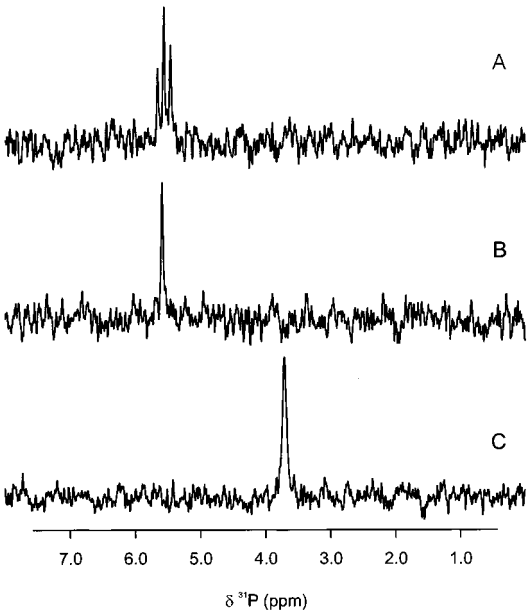

FIGURE 5: ^{31}P NMR spectra of bL18 at 35 °C under different ionic conditions. (A) In 0.2 M NaCl, 8 M urea, 20 mM Tris-HCl, pH 8. (B) In 0.2 M NaCl, 20 mM Tris-HCl, pH 8. (C) In 0.2 M NaCl, 5 mM $MgCl_2$, 20 mM Tris, pH 6.

(*19*). ^{1}H decoupling resulted in the collapse of the triplet into a singlet resonance, and addition of inorganic phosphate to the sample produced a singlet peak at a different chemical shift (3.9 ppm) (data not shown). Figure 5B shows the ^{31}P NMR spectrum of the folded bL18 protein at pH 8. The $^3J_{POCH}$ is too small to measure in this sample, which indicates that both β-protons are now gauche with respect to the phosphorus (*19*). At pH 6 in the presence of Mg^{2+} (Figure 5C), the ^{31}P NMR resonance of bL18 shifts upfield by 1.3 ppm, an effect that has been observed in other proteins (*20*).

The Phosphoserine of bL18 Is Important for 5S rRNA Binding. To find out if the phosphoserine of bL18 influences its binding to 5S rRNA, the protein was incubated with phosphatase, and aliquots were taken at several times. The protein was precipitated with acetone (50%), and the supernatant was assayed for inorganic phosphate. After dialysis of the precipitated protein into a renaturing buffer, a 5S rRNA binding assay was run, with the results shown in Figure 5. Incubation with alkaline phosphatase for up to 18 h did not affect the 5S rRNA binding properties of bL18 (Figure 6A, bottom, lanes 5 and 6), but no inorganic phosphate was released either (Figure 6A, top), indicating that bL18 is a poor substrate for alkaline phosphatase. When acid phosphatase was used, however, the result was different (Figure 6B). After incubation with this enzyme, bL18 no longer binds to 5S rRNA (Figure 6B, bottom, lanes 5 and 6), and phosphate assays showed that >90% of the bL18 present was dephosphorylated (Figure 6B, top). When bL18 was incubated under the same conditions in the absence of phosphatases, the amount of P_i released was insignificant (Figure 6A and 6B, top), and bL18 retained its capacity to bind 5S rRNA (Figure 6A and 6B, bottom, lanes 3 and 4).

To rule out the possibility that the phosphorylation of bL18 observed is an artifact of the overexpression system used to produce the protein, the same experiment was done using L18 isolated from *E. coli* ribosomes (EL18), and the results were similar (data not shown). Incubation of 7.7 nmol of EL18 with acid phosphatase for 4 h resulted in the release of 6.4 nmol of P_i, and the dephosphorylated protein did not bind to 5S rRNA. Only 0.8 nmol of P_i was released when the same amount of EL18 was incubated under the same conditions in the absence of acid phosphatase, and the EL18 so-treated bound to 5S rRNA. Because of the low solubility of EL18, ^{31}P NMR spectra were not obtained for this protein.

Titration of bL18's Phosphoserine Correlates with Its Conformation. If the state of protonation of bL18's phosphoserine affects its conformational state, the chemical shift of the phosphorus of its phosphate group should titrate in parallel with its CD spectrum. The effect of pH on the bL18 phosphorus chemical shift was measured by ^{31}P NMR, and the titration curves that resulted are shown in Figure 7. In the absence of Mg^{2+}, the pK_a of phosphorus cannot be accurately determined due to protein precipitation below pH 6.5, but it is clear that the phosphoserine of bL18 is fully deprotonated only at pHs above 7.5. However, in the presence of Mg^{2+}, the protein does not precipitate below pH 6.5, and the apparent pK_a of its phosphate group is ~5.2. Thus, in the presence of Mg^{2+}, the phosphoserine in bL18 is predominantly dianionic at pH 6.0, but it does not reach that condition until roughly pH 8 in the absence of Mg^{2+}. Thus, the titration of the phosphoserine does indeed correlate with the conformation of bL18, which suggests that a dianionic phosphoserine is necessary for proper protein folding.

DISCUSSION

The results discussed above demonstrate that ribosomal protein bL18 contains a phosphoserine critical for its function at physiological pH. The simplest hypothesis is that the functional importance of this residue derives from its effect on conformation. The CD and NMR data show that in the absence of Mg^{2+}, bL18 is well-folded only at pHs above the physiological range, when the phosphoserine is fully deprotonated. In the presence of Mg^{2+}, the pK_a of the phosphate drops enough to ensure that bL18 is folded at physiological pHs. We cannot exclude the possibility that other groups in bL18 titrating in the same pH range cause a conformational change that affects the chemical shift of the phosphate group in question. However, the correlation between the conformation of bL18 and the pK_a of this phosphate group, both in the presence and in the absence of Mg^{2+}, suggests the effect is direct. It is likely that the dependence of L18's capacity to bind 5S rRNA at neutral pH on the presence of an intact phosphoserine residue is a manifestation of that residue's impact on the protein conformation.

Alignment of the sequences of bL18 and the L18's of other eubacteria shows that there is only one serine residue that is 100% conserved (Figure 1) (*7*), namely, Ser57 in the bL18 sequence. This could be the serine that is phosphorylated, but beyond the fact of its conservation, the evidence supporting this hypothesis is no more than suggestive. The sequence around this serine in the L18 from *B. stearothermophilus* and from many other eubacteria L18's (Figure 1, see *7*) is the same as the sequence recognized by a eukaryotic protein kinase, casein kinase II (CKII), which recognizes the motif -X-Ser/Thr-X-X-Asp/Glu- (*21*). Interestingly, the ribosomal protein that binds 5S rRNA in humans, L5 (*22*),

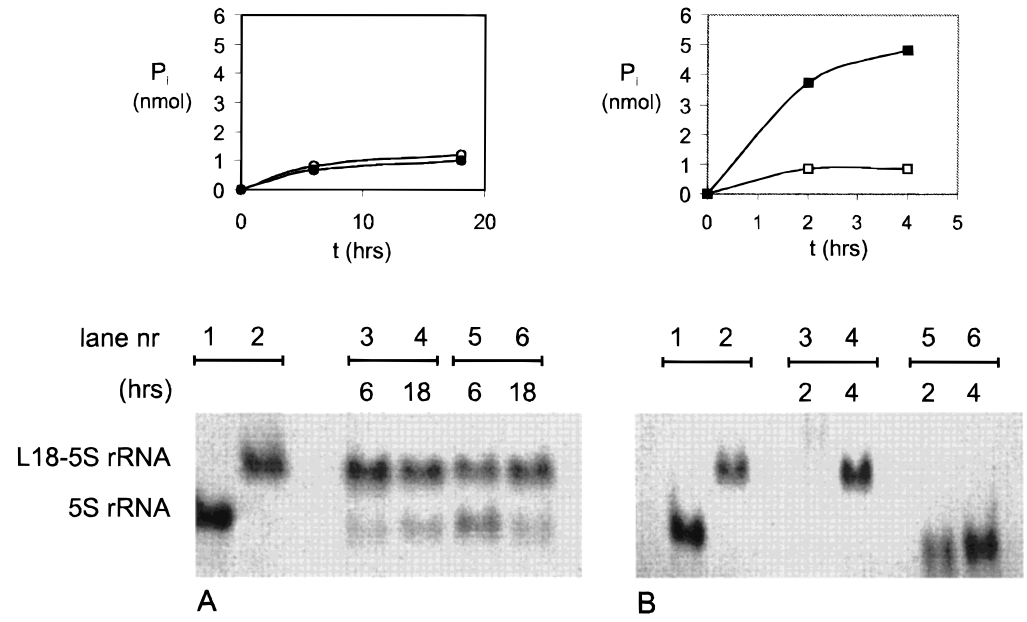

FIGURE 6: Binding of bL18 to 5S rRNA after digestion with alkaline phosphatase (A) or acid phosphatase (B). Bottom panels on both sides: lanes 1 and 2 are references for 5S rRNA and 5S rRNA−bL18 complex, respectively; lanes 3−6 show the 5S rRNA binding properties of bL18 incubated in the absence (lanes 3 and 4) or in the presence (lanes 5 and 6) of the phosphatase in question. Top panels on both sides: P_i release during digestion of bL18 with alkaline phosphatase (○, ●) and acid phosphatase (□, ■). Open symbols are data from controls incubated in the absence of phosphatase, and closed symbols are data from samples incubated with phosphatase. (See Materials and Methods for details.)

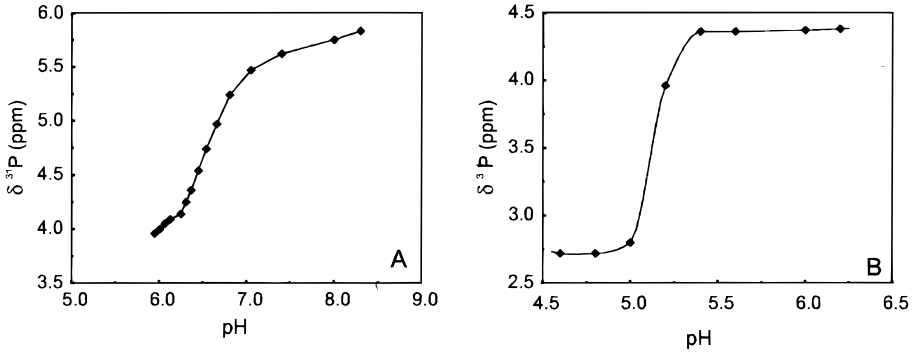

FIGURE 7: Phosphoserine ^{31}P chemical shifts versus pH at 35 °C for bL18 in the absence (A) and presence (B) of $MgCl_2$. Solid lines represent the best fit to the data.

which includes sequences homologous to prokaryotic L18 (*23*), interacts with the β subunit of CKII (*24*), and its chicken homologue binds to protein phosphatase PP1 (*25*). Although it is not known if either human or chicken L5 is phosphorylated in vivo, the corresponding 5S rRNA binding ribosomal protein in yeast, called YL3, L1a, or L5, does contain a phosphoserine residue (*26*), and other components of the eukaryotic translational machinery are phosphorylated, e.g., ribosomal protein S6 and several initiation factors. The functional significance of these phosphorylations is not understood (*27*).

It is only recently that protein kinases such as those found in eukaryotes have been discovered in bacteria (*28*), and as far as we know, no analogue of CKII has yet been identified in *E. coli* or *B. stearothermophilus*. Nevertheless, protein phosphorylation, which was once considered characteristic of eukaryotes, does occur in prokaryotes (*29*), and two chloroplast ribosomal proteins (S6 and L18) appear to contain phosphoserines (*30*).

Since phosphorylated forms of L18 (or L5 in eukaryotes) are present in many organisms, the question of their function arises. In many systems, phosphorylation is used to control the biological activity of proteins (*31*), and phosphorylation can result in significant conformational changes (*32*). Could it be that bacteria use phosphorylation of L18 to control either translation or ribosome assembly?

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