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A Fourier-Transform Infrared Spectroscopic Study of the Phosphoserine Residues in Hen Egg Phosvitin and Ovalbumin†

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ABSTRACT: A Fourier-transform infrared spectroscopic study of hen egg phosvitin and ovalbumin has been carried out. Bands arising from monoanionic and dianionic phosphate monoester [Shimanouchi, T., Tsuboi, M., & Kyogoku, Y. (1964) *Adv. Chem. Phys. 8*, 435-498] can be identified easily in the 1300-930 cm^{-1} region in spectra of solutions of *O*-phosphoserine and phosvitin, a highly phosphorylated protein. On the other hand, spectra of ovalbumin show a relatively strong absorption above 1000 cm^{-1} arising from the protein moiety. Below 1000 cm^{-1}, a single band at 979 cm^{-1} is observed; this band is not present in spectra of dephosphorylated ovalbumin, and therefore, it has been assigned to the symmetric stretching of the phosphorylated Ser-68 and Ser-344 in the dianionic ionization state. In addition, bands arising from symmetric and antisymmetric stretchings of the monoanionic ionization state, and from the antisymmetric stretching of the dianionic state, can be detected above 1000 cm^{-1} in difference spectra of ovalbumin minus dephosphorylated ovalbumin. The effect of pH on the infrared spectra of *O*-phosphoserine, phosvitin, and ovalbumin is consistent with the phosphoserine residues undergoing ionization with p*K* values about 6. This study demonstrates that Fourier-transform infrared spectroscopy can be a useful technique to assess the ionization state of phosphoserine residues in proteins in solution.

We have recently shown that Fourier-transform infrared (FTIR)[1] spectroscopy can be a valuable technique to determine the ionization state of protein-bound phosphoryl groups for coenzyme-dependent enzymes (Sanchez-Ruiz & Martinez-Carrion, 1986).

Information regarding ionization states of bound phosphoryl groups may, in many cases, not be obvious on the basis of ^{31}P NMR chemical shift studies, as the phosphorus chemical shift is sensitive to factors other than the charge on the phosphoryl group, such as the O-P-O bond angle [Gorenstein, 1975; for

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[1] Abbreviation: FTIR, Fourier-transform infrared.

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a review of ^{31}P NMR studies on protein-bound phosphate groups, see also Vogel (1984)]. Thus, chemical shifts for protein-bound phosphoryl groups often differ from those obtained for model compounds in solution (Chlebowski et al., 1976), and in some cases, chemical shift/pH titration curves have been shown to be due to the influence of a nearby residue rather than to actual ionization of the phosphoryl group (Gorenstein et al., 1976; Porubcan et al., 1979; Sanchez-Ruiz & Martinez-Carrion, 1986).

In the infrared spectrum of cytosolic holoaspartate aminotransferase, we could identify the band arising from the symmetric stretching of the dianionic phosphate monoester of bound pyridoxal phosphate (Sanchez-Ruiz & Martinez-Carrion, 1986). The integrated intensity of this band did not change with pH, suggesting that the phosphate group of the bound coenzyme remains mostly dianionic over the pH range 5.3–8.6. Thus, FTIR spectroscopy could be used to assess the ionization state of protein-bound phosphoryl groups, and it appears interesting to test the applicability of this technique to other types of phosphorylated protein groups, such as those containing phosphoserine residues.

Phosvitin is the major phosphorylated protein in egg yolk. It has a molecular weight of 34000 (Taborsky, 1974), and its sequence of 216 residues includes 123 serines, many of these being phosphorylated (Byrne et al., 1984). ^{31}P NMR spectra of phosvitin show a broad band arising from the overlapping resonances of the phosphoserine residues, with limiting chemical shifts at low and high pH being similar to those determined for model phosphate monoesters in solution (Vogel, 1983). However, the chemical shift/pH titration curves are clearly biphasic, showing a larger inflection at about pH 6 and a smaller one at about pH 10. The reason for this behavior is unclear. A previous FTIR analysis of phosvitin (Renugopalakrishnan et al., 1985) was primarily concerned with the secondary structure of the protein, and it also reports the presence of phosphate group vibrations in the solid state at 980 and 920 cm^{-1} but does not follow the pH dependence of the FTIR spectrum in solution.

By contrast, hen egg white ovalbumin is a monomeric protein comprised of 385 amino acid residues with a molecular weight of 43000. Ser-68 and Ser-344 are phosphorylated as result of a posttranslational modification (Nisbet et al., 1981). Recently, two ^{31}P NMR studies on the phosphoserine residues of ovalbumin have appeared in the literature (Vogel & Bridger, 1982; Goux & Venkatasubramanian, 1986). The resonances arising from the two phosphoserine residues were well resolved, and their pH titration behavior resembled, in terms of pK value (about 6) and size of the chemical shift change, the behavior of phosphoserine in solution. In addition, the correlation times, calculated from the frequency dependence of the line widths, were less than half the overall correlation time expected for a protein the size of ovalbumin (Vogel & Bridger, 1982). These results, and the fact that both phosphoserine residues are susceptible to phosphatase digestion (Vogel & Bridger, 1982), indicate that both residues are exposed to the solvent and can undergo ionization with a pK of about 6. This fact makes hen egg ovalbumin an excellent model to explore the applicability of FTIR spectroscopy to the study of individual phosphoserine residues in proteins. In this report, we present a FTIR spectroscopic study of the two phosphorylated proteins hen egg phosvitin and ovalbumin.

Experimental Procedures

Phosvitin and *O*-phosphoserine were obtained from Sigma Chemical Co. and used without further purification. The concentrations of phosvitin solutions given refer to milligrams of the commercial product per milliliter.

Ovalbumin was prepared from egg whites by the sodium sulfate method (Kekwick & Cannan, 1936) and was stored as a lyophilized powder. The protein was judged to be pure from its behavior in sodium dodecyl sulfate–acrylamide gel electrophoresis.

Ovalbumin shows heterogeneity with respect to the degree of phosphorylation (Linderstrom-Land & Ottesen, 1949). In order to remove this heterogeneity, the protein was further purified according to the procedure described by Goux and Venkatasubramanian (1986). Briefly, the protein was applied to a column of DEAE-cellulose in 10 mM sodium phosphate buffer, pH 7, and eluted with linear concentration gradient (10–100 mM) of sodium phosphate buffer. The elution profile obtained was virtually identical with the one described by these authors; thus, three major protein fractions were eluted from the column: fractions I, II, and III, in order of increasing elution volume. On the basis of ^{31}P NMR spectra, Goux and Venkatasubramanian identified fraction III as ovalbumin with both Ser-344 and Ser-68 phosphorylated and the minor fractions, I and II, as protein phosphorylated only at Ser-68.

Completely dephosphorylated ovalbumin was prepared by treatment of fraction III with potato acid phosphatase (from Sigma Chemical Co.) according to Vogel and Bridger (1982). Protein concentrations were measured spectrophotometrically by using the known extinction coefficient (Tomimatsu, 1965).

Infrared spectra of protein solutions, at room temperature and 4 cm^{-1} resolution, were obtained in a Sirius 100 (Mattson Instruments) Fourier-transform infrared spectrometer equipped with a liquid nitrogen cooled mercury–cadmium–teluride detector; 1-mm-thick calcium fluoride windows and 25-μm spacers were used in all the experiments. Protein concentrations were 4, 60–80, and 110–120 mg/mL for phosvitin, ovalbumin fraction III, and ovalbumin fraction I, respectively. The aqueous solvent was 50 mM KCl, and the pH values were adjusted with 100 mM sodium arsenate buffer.

At the concentrations used in experiments with ovalbumin, the peak height of the band due to the symmetric stretching of the dianionic phosphate monoester (see Results) was about 0.005 absorbance unit. This band appears over a strong (about 1 absorbance unit) but smooth absorption due to the aqueous solvent. Therefore, in order to observe the phosphate ester band clearly after water subtraction, noise levels below 0.001 absorbance unit are needed. Problems in the detection of small bands as a consequence of the high dynamic range of the interferogram have been reported (Griffiths & de Haseth, 1986). Reduction of the dynamic range of the interferogram by using an optical filter (provided by Mattson Instruments) that blanked essentially all the radiation above 2000 cm^{-1}, combined with a high value (×4) of the amplifier gain, resulted in a substantial improvement of the signal to noise ratio. Under these conditions, noise levels below 0.001 in the region of interest could be obtained after accumulation and Fourier transformation of 800 interferograms. The speed of the moving mirror was 2.53 cm/s, and data were acquired in both movement directions (backward and forward) of the mirror, the total scan time being 2 min, 13 s.

Two procedures were followed to obtain the final solvent-corrected spectra: (1) 800 interferograms for the sample and for an air background were accumulated and Fourier transformed. The resulting spectra were ratioed and transformed logarithmically by a computer to give an absorbance spectrum of the sample. Afterward, the absorbance of the aqueous solvent was subtracted, the subtraction criterion being the obtaining of flat base lines or at least clear minima below and

Table I: Wavenumbers of Bands Arising from Monoanionic and Dianionic Phosphate Monoesters

	characteristic positions[a] (cm^{-1})	O-phosphoserine[b] (cm^{-1})	phosvitin[b] (cm^{-1})	ovalbumin[b] (cm^{-1})
dianionic degenerate stretching	1100	1091	1097	~1090[d]
dianionic symmetric stretching	980	979	981	979
monoanionic symmetric stretching	1080	1085	1082	~1080[d]
monoanionic antisymmetric stretching	1230	1194	~1190[c]	~1200[d]

[a] Shimanouchi et al. (1964). [b] This work. [c] Protein interferences preclude the precise calculation of the position of this band (see Figure 2). [d] Calculated from difference spectra, diphosporylated ovalbumin (fraction III) minus dephosphorylated ovalbumin (see Figure 5).

above the band assigned to the symmetric stretching of the dianionic phosphate monoester [see Sanchez-Ruiz and Martinez-Carrion (1986)]. (2) An absorbance spectrum was obtained as in procedure 1, but in this case, the background consisted of a sample of the aqueous solvent. This produces water oversubtraction, as the concentration of water is expected to be higher in the aqueous solvent (50 mM KCl) than in the protein solutions. Therefore, the spectrum of the aqueous solvent was added according to the above-mentioned criterion.

Method 2 was employed in most cases, because a small amount of interferogram clipping was usually observed in the background interferograms of method 1 at the high amplifier gain used (×4). However, virtually identical spectra and comparable noise levels were obtained with both procedures in most instances.

The spectrum of the solvent was obtained by following procedure 1 with a lower amplifier gain (×2) to avoid background interferogram clipping. Prior to subtraction, this spectrum was smoothed by using a 25-point Savitsky–Golay algorithm. No smoothing was applied to protein or O-phosphoserine spectra.

Band positions were calculated by polynomial fitting to the three highest points. They appeared to be reproducible to about 0.2–0.3 cm^{-1}. Values given in Table I have been rounded to the next wavenumber. Integrated intensities of the band assigned to the symmetric stretching of the dianionic phosphate were calculated as previously described (Sanchez-Ruiz & Martinez-Carrion, 1986).

Results and Discussion

Infrared Spectra of O-Phosphoserine in Solution. Monoanionic and dianionic phosphate monoesters give rise to two bands each in the infrared spectrum (Shimanouchi et al., 1964; see Table I). These bands can be easily identified in infrared spectra of O-phosphoserine in solution (Figure 1) on the basis of (a) their wavenumbers (see Table I) and (b) their pH titration behavior; thus, the intensity of the bands assigned to the phosphate ester in Figure 1 changes with pH according to the pK value (about 6) expected for a phosphate monoester.

It is worth noting that infrared spectral bands of O-phosphoserine appear in a spectral region (1300–930 cm^{-1}) very similar to the one previously reported for pyridoxal phosphate (Sanchez-Ruiz & Martinez-Carrion, 1986), as would be expected if the phosphate monoester bands are the dominant features in this spectral region.

Infrared Spectra of Phosvitin in Solution. Infrared spectra of hen egg phosvitin in the region 1300–930 cm^{-1} closely resemble those obtained for O-phosphoserine, and, therefore,

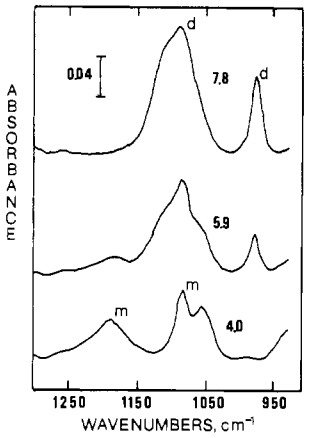

FIGURE 1: Infrared spectra of O-phosphoserine at the indicated pHs. Concentration is, in all cases, 50 mM. Bands assigned to monoanionic and dianionic ionization states have been labeled m and d, respectively.

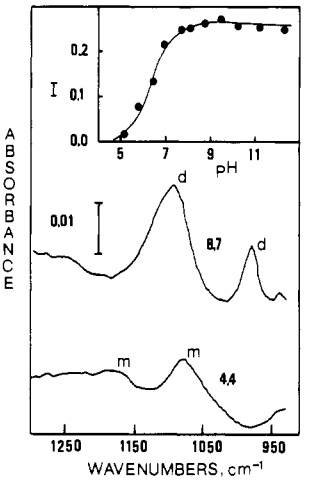

FIGURE 2: Infrared spectra of phosvitin at the indicated pHs. The protein concentration is, in both cases, 4 mg/mL. Bands assigned to monoanionic and dianionic ionization states have been labeled m and d, respectively. (Inset) Effect of pH on the integrated intensity (I, cm^{-1}) of the band assigned to the symmetric stretching of the dianionic phosphate monoester.

the identification of phosphate monoester bands is straightforward (Figure 2 and Table I). Interferences due to protein groups appear to be minor and are apparent only in the 1300–1200 cm^{-1} region (Figure 2). This situation could be expected in a highly phosphorylated protein, such as phosvitin.

Vogel (1983) has investigated the ionization behavior of the phosphoserine groups in phosvitin by using ^{31}P NMR spectroscopy. A broad band arising from overlapping resonances and some minor peaks were observed. The high- and low-pH limiting chemical shifts were similar to those determined for O-phosphoserine (Vogel & Bridger, 1982); however, the chemical shift/pH titration curves for the broad resonance band and two of the minor resonance bands were clearly biphasic, showing a large inflection at about pH 6 and a minor one at about pH 10. Two interpretations of these results were offered (Vogel, 1983). (1) The larger inflection is a result of the ionization of the phosphoserine residues, while the minor one is due to the fact that the chemical shift is able to sense the ionization of basic residues in the protein. (2) Some of the phosphoserine groups have an anomalously high pK which would not be unusual for a highly charged protein (Zuiderweg et al., 1979).

Infrared spectra of phosvitin reflect the ionization of the phosphoserine residues. Thus, the only bands present in spectra obtained at pH values below 5 are those assigned to mo-

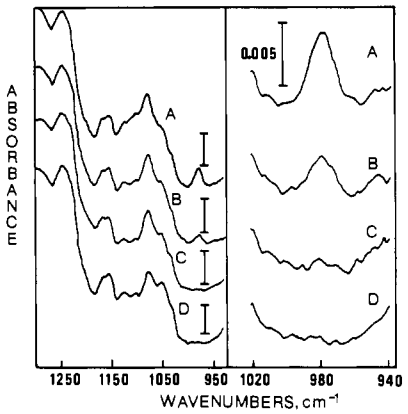

FIGURE 3: (Left) (A–C) Infrared spectra of diphosphorylated ovalbumin (fraction III) at the following pHs and concentrations: (A) pH 9, 73 mg/mL; (B) pH 6.2, 67 mg/mL; (C) pH 4.8, 62 mg/mL. (D) Infrared spectra of dephosphorylated ovalbumin, pH 6, 86 mg/mL. In all cases, the scales shown correspond to 0.01 absorbance unit. (Right) Blowup of the 1020–940 cm^{-1} region. The absorbance scale has been referred to 2 mM ovalbumin.

noanionic phosphate, while spectra at pH values above 8 show bands attributed to the dianionic ionization state (Figure 2). At intermediate pH, the spectra can be easily interpreted as a mixture of both ionization states.

The infrared spectra of phosvitin do not significantly change in the pH range 8–12, and a plot of the integrated intensity of the band assigned to the symmetric stretching of the dianionic phosphate monoester versus pH is monophasic. Therefore, the FTIR spectra of phosvitin do not indicate ionization of phosphoserine residues in that pH range. If the high-pH inflections observed in the phosphorus chemical shift/pH titration curves (Vogel, 1983) were due to ionization, the above-mentioned integrated intensity versus pH profile should be biphasic, showing an increase in the pH range 8–12. Yet, by FTIR, only a slight decrease is detected, which is within the experimental uncertainty. Thus, our infrared data support interpretation 1 (see above); that is, there appears to be no significant population of phosphoserine residues with abnormally high p*K* values, and the minor inflection observed in ^{31}P chemical shift/pH titration curves most likely reflects the ionization of basic residues in the protein.

Infrared Spectra of Ovalbumin. Figure 3 shows infrared spectra of diphosphorylated ovalbumin (fraction III; see Experimental Procedures) at several pH values. The spectrum at pH 9 is remarkably similar to the one reported for cytosolic aspartate aminotransferase (Sanchez-Ruiz & Martinez-Carrion, 1986). A relatively strong absorption is observed at about 1000 cm^{-1}, but only a single band at 979 cm^{-1} (975–977 cm^{-1} in cytosolic aspartate aminotransferase) is detected in the 1000–930 cm^{-1} region. Spectra of dephosphorylated ovalbumin show a similar absorption above 1000 cm^{-1} (Figure 3); however, the 979 cm^{-1} band is absent even at a pH value of 9. Accordingly, we have assigned this band to the symmetric stretching of the dianionic phosphate (see Table I). Infrared spectra of monophosphorylated ovalbumin (fraction I) provide further support for this assignment: spectra of fraction I at pH values above 7 are similar to those for fraction III; however, the integrated intensity of the band at 979 cm^{-1}, per mole of protein, for diphosphorylated ovalbumin is twice as large as the one determined for monophosphorylated ovalbumin (results not shown).

The main difference between infrared spectra of cytosolic aspartate aminotransferase and those of ovalbumin is that, in the first case, the integrated intensity of the band assigned to the symmetric stretching of the dianionic phosphate remained

Table II: p*K*'s of Phosphoserine Residues Determined from ^{31}P NMR Chemical Shift and Fourier-Transform Infrared Spectroscopic Studies

		^{31}P NMR chemical shift (ppm)		
		a	*b*	FTIR[c]
O-phosphoserine		5.75		6.0
diphosphorylated ovalbumin	Ser-344	6.00	6.22	6.2[d]
	Ser-68	6.04	6.16	
monophosphorylated ovalbumin	Ser-68	5.60[e]	5.9[f]	

[a] Vogel and Bridger (1982). [b] Goux and Venkatasubramanian (1986). [c] This work. [d] A single band arising from both residues is observed at 979 cm^{-1}. [e] Monophosphorylated ovalbumin obtained by treatment of commercial ovalbumin with alkaline phosphatase. [f] Fraction I of the DEAE-cellulose elution profile (see Experimental Procedures).

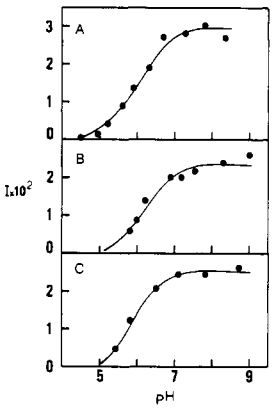

FIGURE 4: Effect of pH on the integrated intensity (I, cm^{-1}) of the band assigned to the symmetric stretching of the dianionic phosphate monoester. (A) *O*-phosphoserine; (B) ovalbumin fraction III (diphosphorylated); (C) ovalbumin fraction I (monophosphorylated). Integrated intensity values have been referred to 1 mM phosphate monoester.

constant in the pH range 5.3–8.6 (Sanchez-Ruiz & Martinez-Carrion, 1986), while for ovalbumin the intensity does change with pH, the band being practically absent at about pH 5 (Figure 3). In fact, integrated FTIR spectral intensity/pH profiles for *O*-phosphoserine and diphosphorylated and monophosphorylated ovalbumin indicate that, in these three cases, the phosphate monoester groups ionize with p*K* values of about 6. The values calculated from nonlinear least-squares fit of the intensity data are given in Table II.

As previously indicated (Sanchez-Ruiz & Martinez-Carrion, 1986), integrated intensity calculations should be regarded as "semiquantitative", due, among other factors, to the use of linear base lines and the possible presence of small buried bands. Two lines of evidence suggest, however, that no large errors are introduced by the integrated intensity calculation procedure: (1) there is an acceptable agreement between the p*K* values calculated from ^{31}P NMR chemical shift/pH titration curves and those determined by FTIR; (2) there is also a good agreement between the high-pH limiting integrated intensities per mole of phosphate monoester for *O*-phosphoserine and mono- and diphosphorylated ovalbumin (see Figure 4).

In addition to the above-mentioned pH dependence of the intensity of the band at 979 cm^{-1}, minor changes in the region 1100–1200 cm^{-1} are observed as the pH is lowered (see Figure 3). In order to look into the origin of these changes, difference spectra of diphosphorylated ovalbumin minus dephosphorylated ovalbumin, at low and high pH, were obtained (Figure 5). These difference spectra closely resemble spectra of *O*-phosphoserine and phosvitin at similar pH values (compare

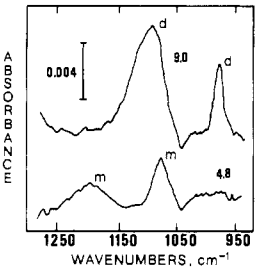

FIGURE 5: Difference spectra, diphosphorylated ovalbumin (fraction III) minus dephosphorylated ovalbumin, at the indicated pHs. Diphosphorylated ovalbumin concentrations were 73 mg/mL (pH 9) and 62 mg/mL (pH 4.8). The subtraction criterion was to cancel the strong protein band at 1250 cm^{-1} (see Figure 2). Bands assigned to monoanionic and dianionic phosphate monoester have been labeled m and d, respectively.

Figures 1 and 2 with Figure 5). It appears, therefore, that the change detected in the spectral region 1100–1200 cm^{-1} also reflects the ionization of the phosphoserine residues, and, accordingly, we have assigned the bands observed in the difference spectra to the symmetric and antisymmetric stretchings of the dianionic and monoanionic phosphate monoester (Table I).

It is of interest that, in our previous FTIR study of cytosolic aspartate aminotransferase (Sanchez-Ruiz & Martinez-Carrion, 1986), difference spectra (holoenzyme minus apoenzyme) in the 1250–1000 cm^{-1} region were difficult to interpret, as no clear base lines were obtained. Two reasons for this can be offered: (1) the ratio "number of phosphate monoester groups/weight of protein" is higher for ovalbumin than for the aminotransferase; (2) removal of the coenzyme from holaspartate aminotransferase may be accompanied by a conformational alteration that might result in a change in the protein absorption in the region 1250–1000 cm^{-1}. The latter is less likely to happen upon ovalbumin dephosphorylation, as the phosphoserine residues have been shown to be exposed to the solvent (Vogel & Bridger, 1982) and are not expected to interact strongly with other protein residues. Thus, although the interpretation of ^{31}P NMR chemical shift data in terms of ionization states is not straightforward in many instances, there seems to be little doubt that in ovalbumin (Vogel & Bridger, 1982) the pH titration reflected NMR behavior of the ionization of two phosphoserine residues.

CONCLUSIONS

The aim of this work was to show that FTIR spectroscopy can be used as a general technique to determine the ionization state of protein-bound phosphoryl residues. This was suggested by a previous FTIR study of a bound coenzyme 5′-phosphate ester in cytosolic aspartate aminotransferase (Sanchez-Ruiz & Martinez-Carrion, 1986). Now we show that this technique can be useful in the study of phosphoserine residues in proteins. In addition, we show that spectra with signal to noise ratios sufficient to detect phosphate monoester bands can be obtained in small samples at phosphate concentrations of about 3 mM and in comparatively short times (2 min).

Thus, all phosphate monoester bands could be easily identified in phosvitin, a protein with more than a hundred phosphoserine residues. More significantly, in ovalbumin, a protein with a low degree of phosphorylation, the band assigned to the symmetric stretching of the dianionic phosphate monoester can be detected in the original spectra, and other phosphate monoester bands could be identified in difference spectra, diphosphorylated ovalbumin minus dephosphorylated ovalbumin. The effect of pH on the FTIR spectra indicates that the phosphoserine residues undergo ionization with pK values about 6. This is consistent with ^{31}P NMR studies (Vogel & Bridger, 1982) in which chemical shift/pH titration curves with inflection points at about pH 6 and large chemical shift changes (about 4 ppm) were observed.

Finally, some comments regarding the advantages and disadvantages of the FTIR technique when applied to protein-bound phosphoryl groups are warranted. Under our experimental conditions, FTIR does not seem to be able to distinguish between very similar types of protein-bound phosphoryl residues (for instance, phosphorylated Ser-344 and Ser-68 in hen egg ovalbumin). In addition, protein interferences may preclude the detection of some of the phosphate stretching bands; yet, in many cases, information regarding the ionization state of phosphoryl residues can be derived from the intensity of the band arising from the symmetric stretching of the dianionic phosphate monoester alone, which appears in a region where protein interferences are minor. FTIR should be used as a method complementary to NMR in its capability to monitor ionization states. Thus, the comparison between the integrated band intensity versus pH profile for the FTIR bands and the ^{31}P NMR chemical shift/pH titration curve allows for more precise assignment as to whether changes seen in the latter are due to ionization of phosphoryl residues or to the ionization of a nearby residue in the protein that affects the phosphorus chemical shift.

Registry No. Phosphoserine, 407-41-0.

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