Markley, J. L. (1975) *Acc. Chem. Res. 8*, 70–80.
Markley, J. L., & Kato, I. (1975) *Biochemistry 14*, 3234–3237.
Martin, M. L., Delpeuch, J.-J., & Martin, G. J. (1980) *Practical NMR Spectroscopy*, pp 258–259, Heyden, London.
Ogez, J. R., Tivol, W. F., & Benisek, W. F. (1977) *J. Biol. Chem. 252*, 6151–6155.
Penning, T. M., & Talalay, P. (1981) *J. Biol. Chem. 256*, 6851–6858.
Ray, W., Jr., & Koshland, D. E. (1962) *J. Biol. Chem. 237*, 2493–2505.
Sheinblatt, M. (1966) *J. Am. Chem. Soc. 88*, 2845–2848.
Stanier, R. Y., Palleroni, N. J., & Doudoroff, M. (1966) *J. Gen. Microbiol. 43*, 159–271.
Talalay, P., & Wang, V. S. (1955) *Biochim. Biophys. Acta 18*, 300–301.
Tivol, W. F., Beckman, E. D., & Benisek, W. F. (1975) *J. Biol. Chem. 250*, 271–275.
Wang, S.-F., Kawahara, F. S., & Talalay, P. (1963) *J. Biol. Chem. 238*, 576–585.
Weintraub, H., Alfsen, A., & Baulieu, E.-E. (1970) *Eur. J. Biochem. 12*, 217–221.
Weintraub, H., Vincent, F., Baulieu, E.-E., & Alfsen, A. (1973) *FEBS Lett. 37*, 82–88.
Weintraub, H., Vincent, F., Baulieu, E.-E., & Alfsen, A. (1977) *Biochemistry 16*, 5045–5053.
Westbrook, E. M. (1976) *J. Mol. Biol. 103*, 659–664.
Westbrook, E. M., Sigler, P. B., Berman, H., Glusker, J. P., Bunick, G., Benson, A., & Talalay, P. (1976) *J. Mol. Biol. 103*, 665–667.
Wuthrich, K. (1976) *NMR in Biological Research: Peptides and Proteins*, pp 144–146, North-Holland Publishing Co., Amsterdam.
Yamasaki, R. B., Osuga, D. T., & Feeney, R. E. (1982) *Anal. Biochem.* (in press).

Phosphorus-31 Nuclear Magnetic Resonance Studies of the Two Phosphoserine Residues of Hen Egg White Ovalbumin†

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ABSTRACT: Ovalbumin contains two phosphoserine residues that give rise to two well-resolved resonances in a ^{31}P NMR spectrum. Ovalbumin samples that have been digested with a variety of phosphatases may give rise to only one phosphoserine resonance, indicating that one of the two phosphorylated sites is relatively inaccessible for phosphatase action. By comparison of the amino acid sequence of the peptide containing the nonsusceptible phosphate to the overall primary structure, we have assigned the resonances observed (pH 8.3) at 5.0 and 4.75 ppm to phosphoserines-68 and -344, respectively. pH titration behavior and susceptibility of the phosphoserine residues to phosphatases indicate that both are located on the surface of the protein. Both residues have a pK_a = 6.00–6.04. Analysis of the Hill coefficients measured for the pH titrations and the J_{PH} coupling constants indicate that neither residue interacts with other charged groups on the surface of the protein. Frequency dependence of ^{31}P NMR parameters shows that at higher magnetic field strengths the contribution of chemical shift anisotropy to the line width becomes very significant. We have calculated from the field-dependent terms that phosphoserine-344 is mobile with respect to the protein surface but that phosphoserine-68 is more restricted in its motion. The latter is also involved in a pH-dependent conformational change, since it is shielded from hydrolysis by phosphatases at higher pH. A comparison of the amino acid sequence of the phosphoserine-68 site shows that it has a striking homology to the active-site peptides of a wide variety of hydrolytic enzymes. Moreover, a comparison with the primary sequences of casein suggests that both proteins are phosphorylated by a protein kinase that specifically recognizes a Ser-X-Glu peptide.

Ovalbumin is the most abundant protein of egg white, from which it can be purified and crystallized in large quantities (Hofmeister, 1889). Although its properties have been frequently studied as those of a "model protein", very little is known about its biological function (Taborsky, 1974). Ovalbumin synthesis in the chick oviduct is under hormonal control and requires the presence of steroid sex hormones; this control has been extensively studied at the level of transcription as well as translation (Palmiter, 1975). Moreover, this protein has also proved to be of interest to students of eukaryotic gene organization (Breathnach et al., 1977).

The complete amino acid sequence of ovalbumin has been deduced both from the mRNA sequence (McReynolds et al., 1978) and by conventional amino acid sequencing techniques (Nisbet et al., 1981). The protein is a monomer comprised of 385 residues with a molecular weight for the polypeptide chain of 43 000. Unlike most other proteins that are translocated across a membrane, ovalbumin does not possess a hydrophobic N-terminal signal sequence but is believed to contain an internal signal sequence to facilitate its secretion (Lingappa et al., 1979). Most preparations of the protein have detectable heterogeneity, some of which arise from the genetic variants of the protein. The two variants that have been characterized have undergone only one base change, resulting in a replacement between an acid and its amide (Nisbet et al.,

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1981; Ishihara et al., 1981). Other contributions to the heterogeneity mainly arise from differences in the level of posttranslational modifications, such as the N-acetylation of the N-terminal amino acid (Narita et al., 1968) or the heterologous glycosylation of Asn-292 (Tai et al., 1977; Atkinson et al., 1981; Ishihara et al., 1981; Iwase et al., 1981).

A third posttranslational modification is the phosphorylation of serine residues 68 and 344 (Nisbet et al., 1981). Although it has been recognized since the turn of the century that ovalbumin is a phosphoprotein (Osborne & Campbell, 1900), very little is known about the reactions leading to its phosphorylation or dephosphorylation (Taborsky, 1974). Heterogeneity in the electrophoretic behavior of ovalbumin preparations has been attributed to a different extent of phosphorylation (Linderstrøm-Lang & Ottesen, 1949). Phosphatase digestions together with amino acid analysis of peptides resulting from a partial acid hydrolysis of the protein indicated the presence of two nonoverlapping phosphoserine sites (Flavin, 1954; Perlmann, 1955). These observations were confirmed more recently by the peptide sequencing studies of Milstein (1968) and by the completion of the determination of the overall primary structure (Nisbet et al., 1981).

Here we will report on ^{31}P NMR studies of hen egg white ovalbumin. The two phosphoserine residues give rise to resonances that are well resolved in a ^{31}P NMR spectrum and that could be readily assigned. Moreover, we have characterized both residues in terms of metal binding properties, mobility, titratability, environment, and stability toward digestion by a variety of phosphatases.

Experimental Procedures

Ovalbumin was obtained from Sigma Chemical Co. A variety of batches of different purity (V and VI) were used, but no differences were observed for these preparations in ^{31}P, ^{1}H, and ^{13}C NMR spectra. Moreover, all batches were judged pure by behavior as one band on sodium dodecyl sulfate ($NaDodSO_4$)[1]–acrylamide gel electrophoresis. All other enzymes used were purchased from the same source and were used without further purification. PMSF was obtained from Calbiochem. Urea (ultrapure) and Tris were purchased from Schwarz/Mann. All other chemicals were analytical grade. The pH was measured on a Radiometer PHM62 pH meter equipped with a glass electrode. The buffers that were used contained 50 mM Tris-HCl, 1 mM EDTA, and 25% D_2O (Bio-Rad, 99.8%) at pH 8.3. NMR samples were 1.5 mL in 10-mm precision tubes (Wilmad) fitted with Teflon vortex plugs. Most NMR measurements were performed at 28 °C on a Bruker HXS-270 spectrometer at 109.3 MHz. Typical conditions for the experiment were a 60° flip angle and a 2-s recycle time. Proton decoupling during acquisition was used unless otherwise indicated in the text. Frequency-dependent experiments were performed at five different frequencies. Spectrometers used were as previously described (Vogel et al., 1982), and in addition, the spectra at 145.7 MHz were obtained with the Bruker HZ-360 spectrometer at the Dutch National NMR facility at the State University of Groningen, Groningen, The Netherlands. The line widths were measured from the resonances at half-height and were corrected for the additional line broadening introduced by the computer digital filtering. J_{PH} values were estimated (±8%) from resolved curves generated from computer-aided simulations. The nuclear Overhauser enhancements were measured by determining the difference between spectra obtained with proton decoupling only during acquisition and those with decoupling during the whole cycle.

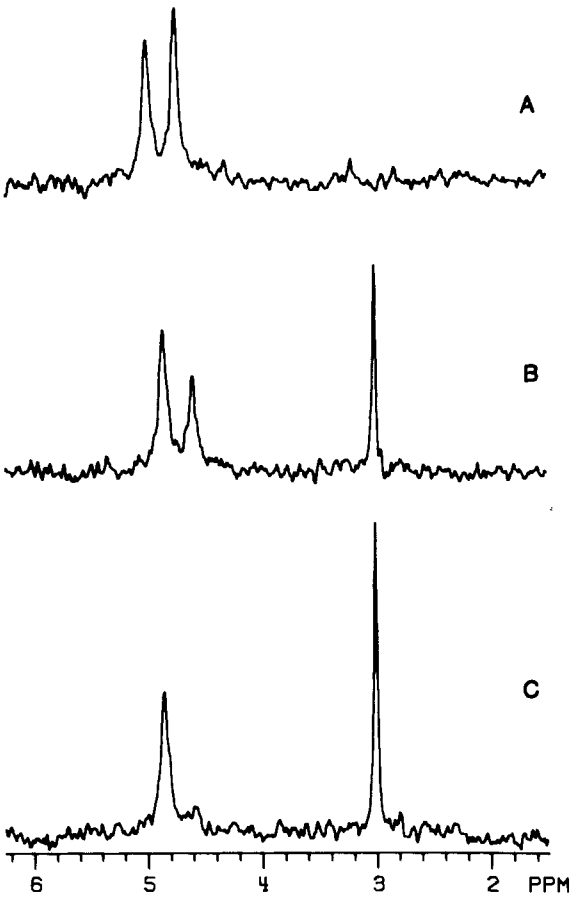


FIGURE 1: Proton-decoupled ^{31}P NMR spectra at 109.3 MHz of hen egg white ovalbumin. (A) Concentration of ovalbumin was 1.0 mM in 50 mM Tris-HCl and 1 mM EDTA, pH 8.3; 500 acquisitions; additional line broadening 2.5 Hz. (B) Same as in (A); 1 h after the addition of 15 mM $MgCl_2$ and 15 units of *E. coli* alkaline phosphatase. The upfield shift of both resonances with respect to those observed in spectrum A is caused by the addition of metal ion. (C) Same as in (B); 20 h after the addition of *E. coli* alkaline phosphatase.

Results

Assignment of the Resonances. Figure 1A shows a proton-decoupled ^{31}P NMR spectrum at 109.3 MHz of a 1 mM solution of ovalbumin at pH 8.3. Similar spectra were obtained over a wide range of concentrations (0.1–2.5 mM). Two well-resolved resonances can be seen at chemical shift positions of 5.00 and 4.75 ppm, respectively (Bock & Sheard, 1975). Integration indicated that the two resonances are of equal intensity. Thus, each resonance represents one phosphoserine residue of the bisphosphoprotein. Early studies had indicated that ovalbumin containing varying amounts of phosphates behaved differently upon electrophoresis and could be separated as the bis-, mono-, and dephosphorylated forms (Linderstrøm-Lang & Ottesen, 1949). Subsequent experiments with several phosphatase preparations have shown that 1 equiv of phosphate could be readily removed to form the monophosphoprotein. Removal of a second equivalent required prolonged incubations (Perlmann, 1955). Figure 1A,C shows a sample of ovalbumin digested with *Escherichia coli* alkaline phosphatase at pH 8.75. The residue corresponding to the resonance originally at 5.0 ppm was resistant to degradation.

[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; Ser*P*, phosphoserine; CIDNP, chemically induced dynamic nuclear polarization; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; Mes, 4-morpholineethanesulfonic acid.

Table I: Percentage Degradation of the Two Phosphoserine Residues of Ovalbumin by Different Phosphatases[a]

		alkaline phosphatase		acid phosphatase	
pH	residue	*E. coli*	calf intestine	potato	wheat germ
8.75	Ser*P*-68	10	30	0	0
	Ser*P*-344	100	100	0	20
7.0	Ser*P*-68	25	75	75	90
	Ser*P*-344	100	100	100	100
5.5	Ser*P*-68	75	90	100	100
	Ser*P*-344	100	100	100	100

[a] All experiments were performed by incubating 1 mM ovalbumin with 20 units of phosphatase for 20 h at 37 °C. Buffers contained 15 mM Mg^{2+} and either 50 mM Tris-HCl (pH 8.75), 50 mM Mes (pH 5.5), or a mixture of both buffers (pH 7.0).

The other resonance at 4.75 ppm has disappeared, with the appearance of a new peak at 3.0 ppm, which could be assigned in terms of chemical shift and pH titratability to inorganic phosphate. Experiments with a variety of other phosphatases (see next section) have confirmed that the Ser*P* resonance on the left is consistently less susceptible to digestion. Thus, the 1 equiv of phosphate that is removed (Perlmann, 1955) all comes from one residue. Studies of partial acid hydrolysates have suggested that the phosphatase-sensitive phosphoserine is present in the peptide sequence Ser*P*-Ala and that the phosphatase-resistant phosphate occurs in the sequence Asp-Ser*P*-Glu-Ile-Ala (Flavin, 1954).[2] By comparing these to the overall sequence, we have assigned the resonance at 5.00 ppm to Ser*P*-68 and the resonance at 4.75 ppm to Ser*P*-344.

Susceptibility to Phosphatase Digestion. At the outset of this study, we performed the digestions with alkaline phosphatases (*E. coli* and calf intestine) at pH 8.75 and with the acid phosphatases at pH 5.5. Thus, the enzymes were incubated close to their respective pH optima, at pHs where ovalbumin is stable. The results (see Table I) suggested that Ser*P*-344 was a good substrate for all phosphates but that Ser*P*-68 could be readily hydrolyzed only by acid phosphatases. This would have been an indication of different specificities for different phosphatases (Neumann, 1968). However, as the other results in Table I show, Ser*P*-68 can be digested at low pH by alkaline phosphatase, and acid phosphatases become less effective in hydrolyzing the phosphate of residue 68 at higher pH. These observations suggest that the substrate ovalbumin may undergo a pH-dependent conformational change and that residue Ser*P*-68 is shielded at higher pH. To substantiate this shielding effect further, we reasoned that peptides, in contrast to the native protein, should be susceptible to *E. coli* alkaline phosphatase digestion at pH 8.75. Thus, in a subsequent experiment, we subjected 1 mM ovalbumin samples to proteolytic cleavage by chymotrypsin and by subtilisin (pH 7.5, 4% protease, 10-h incubation at 37 °C).[3] These samples, together with a control that had been incubated without protease, were treated with 0.5 mM PMSF to inactivate residual protease activity and then were stored at 4 °C for 20 days in order to inactivate the PMSF since compounds such as PMSF may affect the activity of phosphatases

[2] Note that in later studies the sequence of the second peptide has been corrected to Asp-Ser*P*-Ile-Glu-Ala [see Milstein (1968), Taborsky (1974), McReynolds et al. (1978), and Nisbet et al. (1981)].

[3] Proteolysis was confirmed by $NaDodSO_4$–polyacrylamide gel electrophoresis. Trypsin does not attack ovalbumin (Taborsky, 1974), and commercial Pronase is contaminated with a phosphatase (only P_i and no Ser*P* resonances were observed in the ^{31}P NMR spectrum recorded after treatment with Pronase).

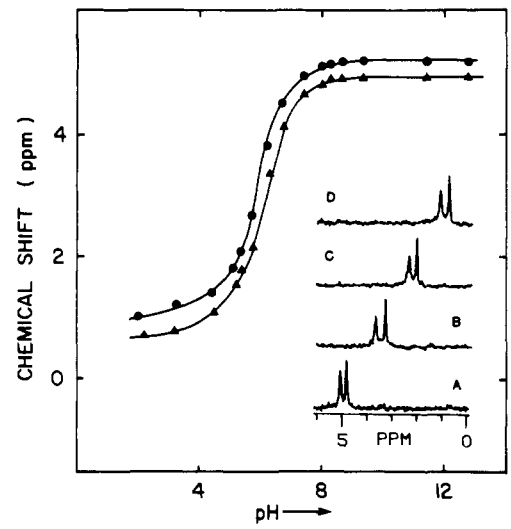


FIGURE 2: ^{31}P NMR pH titration of ovalbumin (1 mM). The spectra shown in the insert were obtained at (A) pH 8.3, (B) 6.2, (C) 5.6, and (D) 3.3. The following symbols are used: (●) Ser*P*-68; (▲) Ser*P*-344. Other conditions were as described in Figure 1.

(Greenberg & Nachmansohn, 1965). Finally, the samples were digested with *E. coli* alkaline phosphatase at pH 8.75 (conditions as in Table I). The resulting ^{31}P NMR spectra showed that in the protease-treated preparations, peptides containing both Ser*P*-68 and Ser*P*-344 were susceptible to phosphatase digestion, but in the control, only Ser*P*-344 was susceptible. This is in agreement with results on peptides by others (Milstein, 1968). Thus, all of these data are in support of the idea that Ser*P*-68 is shielded at high pH and that it becomes accessible at low pH. The results presented in the next section show that the residues have virtually indistinguishable pH titration behavior, rendering this an unlikely explanation for the differential phosphatase sensitivity of residues Ser*P*-68 and Ser*P*-344 (Neumann, 1968). ^{31}P NMR analysis of the time course of digestion by alkaline and acid phosphatases showed that Ser*P*-344 was always more susceptible than Ser*P*-68. This suggests that the former is the more exposed of these two residues.

pH Dependence. Figure 2 shows the pH dependence of the chemical shift positions of the Ser*P* residues of ovalbumin. Clearly, both residues are exposed to solvent. No changes in line width were seen in the course of the titration, indicating that protonated and deprotonated species are in fast exchange on the NMR time scale. Such behavior is typical for a variety of model compounds, but fast exchange is not necessarily a property of phosphoproteins, as was indicated by a split ^{31}P NMR resonance observed at pHs near the pK_a in a titration study of human salivary phosphoproteins (Bennick et al., 1981), for example. The pK_as and Hill coefficients (n) that we have calculated for the phosphoserines-68 and -344 are pK_a = 6.00 and 6.04 and n = 0.94 and 0.87, respectively. These data are typical for phosphoproteins (Vogel, 1982), and they indicate that no charged groups are located proximal to the phosphoryl moiety. In the course of these studies, we have exposed ovalbumin to pH 12.5 at 22 °C. Although after 15 h both Ser*P*'s had been completely hydrolyzed, the rate of disappearance of Ser*P*-344 was higher than that of Ser*P*-68, in keeping with previous reports (Perlmann, 1955).

One possible explanation for the differences in the chemical shifts of the phosphoserine standard (4.5 ppm, pH 8.3) and those of these residues in ovalbumin is that the latter arise from the tertiary structure of the protein. However, the presence of 8 M urea and 25 mM DTT did not result in any obvious

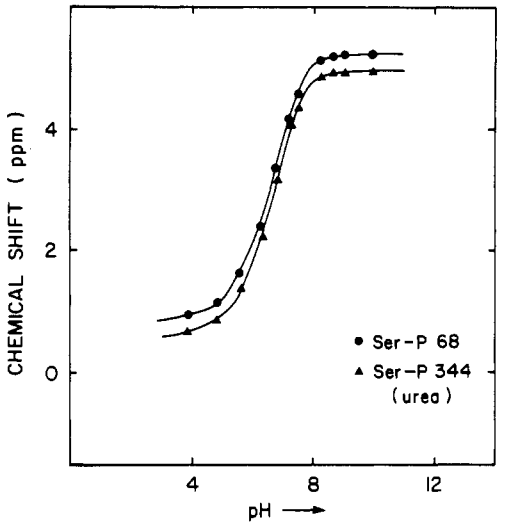


FIGURE 3: pH titration of ovalbumin (1 mM) in 50 mM Tris, 1 mM EDTA, 8 M urea, and 25 mM DTT. Other conditions were as described in Figure 2.

changes in the spectrum taken at pH 8.3. By means of experiments with circular dichroism and ^1H NMR, we have confirmed that the protein was totally unfolded under these conditions (data not shown). We also performed a pH–titration experiment under these denaturing conditions. As the results of Figure 3 indicate, very similar titration curves are obtained for both residues. Although the resonances move closer together at certain pHs than they do in the native protein, they never overlap, and they remain easily recognizable.[4] The pK_as deduced from Figure 3 are 6.61 and 6.56 for residues 68 and 344, respectively. These are higher than those we have measured for the native protein, possibly because of the uncertainty of pH measurement under these conditions. (For example, the pK_as of phosphoserine and P_i were estimated to be 6.10 and 7.40, respectively, when 8 M urea was present but were 5.75 and 6.80, respectively, in the absence of the denaturant.)

Magnesium Titrations. One of the functions suggested for ovalbumin is storage of metal ions (Taborsky, 1974). If the two phosphoryl groups are directly involved in binding metal ions, their chemical shifts would be expected to be influenced by metals, as demonstrated for the human salivary phosphoproteins (Bennick et al., 1981). We find that $MgCl_2$ addition causes upfield shifts for both Ser*P* residues (see Figure 1A,B). At saturating concentrations of metal ion, the resonance for Ser*P*-68 is shifted upfield by 0.25 ppm, and the resonance for Ser*P*-344 is shifted upfield by 0.32 ppm. From double-reciprocal plots of the change in the chemical shifts vs. the concentration of $MgCl_2$, we calculated that the K_d's were approximately 20 and 10 mM for residues 68 and 344, respectively.

Proton–Phosphorus Coupling Constant. Figure 4 shows the difference for proton-coupled and -decoupled spectra of ovalbumin. The signal to noise ratio in the decoupled spectrum is clearly superior, considering that fewer acquisitions were used. The triplet pattern arises from coupling to both protons

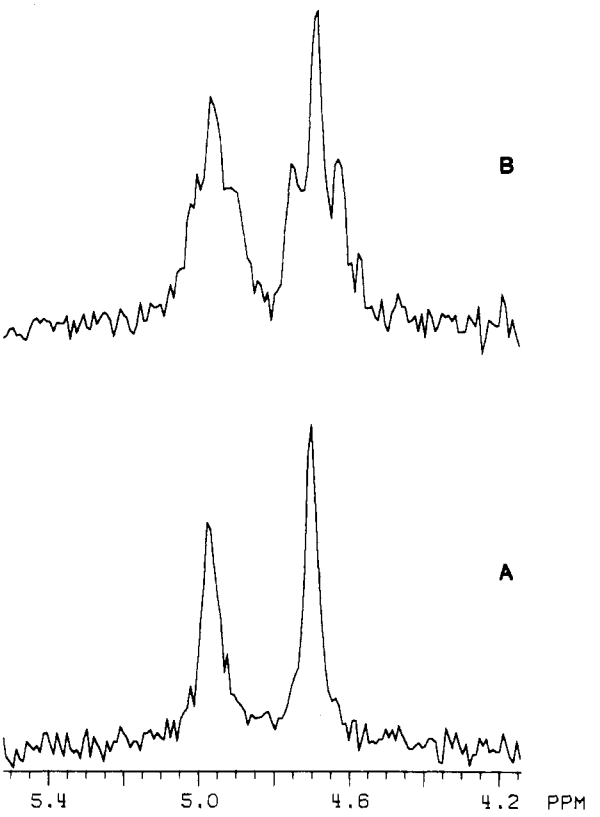


FIGURE 4: Comparison of the proton-coupled (A, 1000 scans) and proton-decoupled (B, 3000 scans) ^{31}P NMR spectra (109.3 MHz) of hen egg white ovalbumin. Additional line broadening due to the computer digital filtering was 0.25 Hz for both spectra.

of the α-methylene group with a coupling constant J_{PH} of 6.5 Hz. Although the coupling is most pronounced for the resonance of residue Ser*P*-344, it is also visible for residue Ser*P*-68. The same value for the coupling constants was obtained from proton-coupled spectra obtained at 81 and 162 MHz and from a sample of a phosphoserine standard. The Ser*P*-68 line width increases when spectra are recorded on a spectrometer operating at 162 MHz to the extent that the proton-induced splitting can no longer be observed (data not shown). Such behavior is diagnostic for the major contribution of chemical shift anisotropy to the relaxation of the phosphorus nucleus. This will be considered in detail in the next section.

Frequency-Dependent Measurements. Comparison of spectra taken at various frequencies clearly indicates that the line widths for both resonances increase at higher magnetic field strength. A similar field dependence has been observed for other phosphoproteins and is caused by the major contribution of chemical shift anisotropy to the relaxation of the phosphorus nucleus [see Vogel et al. (1982)]. Figure 5 shows the results obtained at five different field strengths, indicating that the line width increases linearly with the square of the phosphorus resonance frequency. The intercepts on the y axis show the contribution of field-independent proton–phosphorus dipole–dipole relaxation.[5] Using formulae outlined by Vogel et al. (1982), and substituting an anisotropy term of 110 ppm [Table II of Vogel et al. (1982)], we calculated the correlation times from the chemical shift anisotropy contribution to the

[4] It is noteworthy that Ser*P*-68 had a line width about equal to that of Ser*P*-344 at pH 8.3, indicating that this residue is now less restricted in its motions than it was in the native protein. However, the line widths are such that both residues do not yet resemble a phosphoserine standard. Possibly the area of the protein in which both phosphates are located is held together by charge interactions that remain intact in 8 M urea. Such structures may exist in ovalbumin (Taborsky, 1974).

[5] In principle, proton–phosphorus dipolar interactions can be frequency dependent. However, for molecules of this size at these frequencies, the "non-extreme narrowing limit" is fulfilled, and one can assume frequency independence. See Brauer & Sykes (1981) and Vogel et al. (1982) for a more detailed discussion of this theory and assumption.

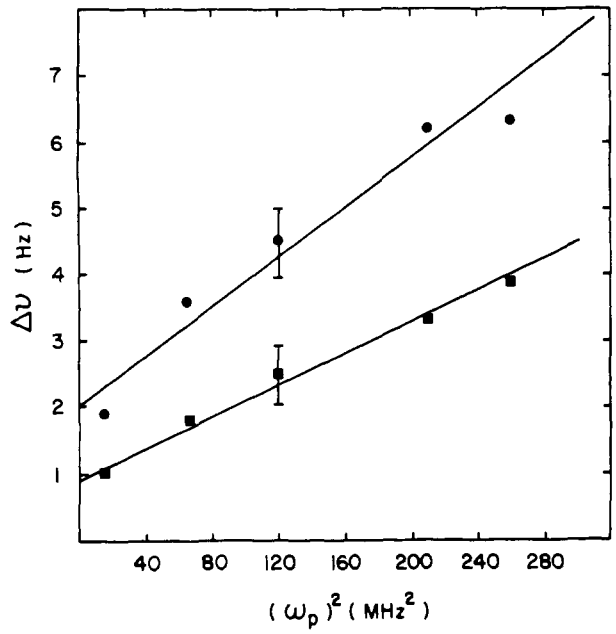


FIGURE 5: Frequency dependence of the line widths measured for Ser*P*-68 (●) and Ser*P*-344 (■). Line widths were measured at half-height, and instrumental line broadening was subtracted. The error bars for the points measured at 109.3 MHz indicate the minimum and maximum values measured at this frequency for a wide variety of samples.

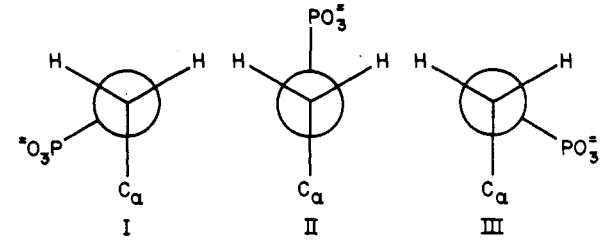
line width for the Ser*P* residues 68 and 344 to be 15 and 8 ns, respectively.[6] We also attempted to determine the nuclear Overhauser enhancement at 109.3 and 145.7 MHz; its contribution was negligible at both frequencies.

Discussion

The overall rotational correlation time for a protein the size of ovalbumin is about 30 ns at the temperature of our experiments (28 °C) (Yguerabide et al., 1970). The rotational correlation times we have determined for the two phosphoryl groups were 8 and 15 ns, respectively. Both residues, especially Ser*P*-344, must have considerable mobility on the protein surface. Our analysis of the time course of phosphatase digestion suggested that Ser*P*-344 is more exposed than Ser*P*-68. Flexibility near Ser*P*-344 had been previously implied by the observation that under certain conditions subtilisin treatment of ovalbumin causes release of only a heptapeptide containing residues 346–352 (Nisbet et al., 1981). It is of interest that we have found that there is a contribution of chemical shift anisotropy to the relaxation of the phosphorus nucleus for these mobile residues. The relative contributions of factors such as chemical shift anisotropy to the relaxation of phosphorus nuclei in small compounds are still controversial (McCain & Markley, 1980; Nanda et al., 1980).

The chemical shifts of both residues, especially Ser*P*-68, deviate somewhat from that of phosphoserine standards. The reason for this deviation is not clear. The pH titrations (Figure 3) did not indicate the presence of proximal charged groups, nor did unfolding of the protein in urea cause a change in the chemical shift. The main effect of the addition of urea was that residue 344 showed more but not complete resemblance to residue 68 as indicated by a change in line width and chemical shift (see Figure 4).

The coupling constants measured for the proton–phosphorus splittings for both residues of the protein are of some interest. The ^{1}H–C–O–^{31}P fragment is known to have an angular dependence of the ^{31}P–^{1}H spin–spin coupling which resembles the well-known Karplus relationship for ^{1}H–^{1}H couplings (Blackburn et al., 1973; Sarma et al., 1973). Three different rotamers are possible as indicated by the following structures.



Structure II is reported to have a J_{PH} of 3 Hz whereas structures I and III each have a coupling constant of 25 Hz (Sarma et al., 1973; Blackburn et al., 1973). The value of 6.6 Hz we measured for the protein-bound residues is the same as that measured for a phosphoserine standard. This value indicates that the rotamer II predominates and that rotation of the phosphoryl moiety is allowed. Thus, this gives further support to our idea that the two phosphoserines of ovalbumin are quite mobile. In contrast, the active-site phosphoserine residue of apoalkaline phosphatase had a J_{PH} of 13 Hz, indicating that rotamer I or III predominates (Chlebowski et al., 1976).

The pH titratability of the Ser*P* residues and their accessibility to phosphatases indicate that both residues are at the surface of the protein. Alkaline phosphatase digestions have been attempted by others for a variety of phosphoproteins; generally, most phosphoenzymes can be dephosphorylated by such treatment (Swarup et al., 1981; Bennick et al., 1981). Thus, the majority of these phosphate residues are probably located on the surface of proteins, as might be expected if the phosphoryl group is attached and removed by enzymes in vivo. By performing phosphatase digestions at different pH values, we have shown that ovalbumin may undergo a pH-dependent conformational change in which Ser*P*-68 becomes shielded at higher pH but is more exposed at lower pH.[7] Since both phosphates are exposed at the surface, it is not surprising that they can bind metal ions. However, the K_d's of 20 and 10 mM for Mg^{2+} binding to the two Ser*P* residues at pH 8.3 do not support a role for these moieties in a special metal binding site, as suggested for human salivary phosphoproteins (Bennick et al., 1981). Dissociation constants of this magnitude are not those expected for proteins designed for storage of metal ions.

Amino acid modification studies of the positively and negatively charged amino acids have indicated that the large majority of such residues are shielded from the solvent (Taborsky, 1974). Thus, the two exposed phosphate residues must make a major contribution to the surface charge of the protein. This may explain why ovalbumin can be so readily separated in its bis-, mono-, and dephosphorylated forms by electrophoresis under nondenaturing conditions (Linderstrøm-Lang & Ottesen, 1949; Perlmann, 1955). It is not clear at present whether this same property can explain the heterogeneity which is sometimes detectable by NaDodSO$_4$–polyacrylamide gel electrophoresis of otherwise apparently homogeneous preparations of ovalbumin (Atkinson et al., 1981).

[6] No corrections were made for the natural line widths or for field inhomogeneity. Standard P line widths did not exceed 0.6 Hz. With the approach used here, this contribution is considered part of the proton–phosphorus dipole–dipole relaxation.

[7] Further support for such a conformational change comes from pH-dependent shielding and deshielding of aromatic residues as implied by photolaser CIDNP experiments (H. J. Vogel and B. D. Sykes, unpublished experiments).

Table II: Comparison of the Amino Acid Sequences of the Ovalbumin Phosphoserine Peptides to Those of Active-Site Peptides of Hydrolytic Enzymes[a]

peptide	sequence
ovalbumin Ser*P*-344	Val-Gly-Ser-Ala
ovalbumin Ser*P*-68	Gly-Asp-Ser-Ile
alkaline phosphatases	
E. coli	Thr-Asp-Ser-Ala
calf intestine	-Asp-Ser-Ala
S. marcescens	Thr-Asp-Ser-Ala
serine proteases	
trypsinogen	Gly-Asp-Ser-Gly
chymotrypsinogen	Gly-Asp-Ser-Gly
elastase	Gly-Asp-Ser-Gly
thrombin	Gly-Asp-Ser-Gly
α-lytic protease	Gly-Asp-Ser-Gly
Streptomyces griseus protease A	Gly-Asp-Ser-Gly
Streptomyces griseus protease B	Gly-Asp-Ser-Gly
Streptomyces griseus trypsin	Gly-Asp-Ser-Gly
subtilisin	Gly-Thr-Ser-Met
aspergillopeptidase	Gly-Thr-Ser-Met
esterases	
carboxylesterase	Gly-Glu-Ser-Ala
acetylcholinesterase	-Glu-Ser-Ala
pseudocholinesterase	Gly-Glu-Ser-Ala
acyl carrier protein *E. coli*	-Asp-Ser-Leu
phosphoglucomutase	Thr-Ala-Ser-His

[a] All data obtained from James et al. (1978) and the CRC Handbook of Biochemistry.

Recently, it was noted that parts of the ovalbumin sequence are homologous to those of the protease inhibitors antithrombin III and α_1-proteinase (Hunt & Dayhoff, 1980). Speculation that the protein could serve as protease inhibitor was not borne out by subsequent experiments (Long & Williamson, 1980). Here we draw attention to another homology: the site around Ser*P*-68 bears a close resemblance to active-site peptides isolated from serine proteases, alkaline phosphatases, esterases, and acyl carrier protein (see Table II). All of these have a glycine followed by an acidic residue and then the "active" serine residue followed by a hydrophobic amino acid. There is no homology evident with phosphoglucomutase and the subtilisin-type protease active sites. Thus, one might speculate that the site around Ser*P*-68 in ovalbumin is a remnant of a possible hydrolytic active site. In this context, it is interesting that the location of an acid residue preceding the Ser*P* is highly conserved. The peptide sequences determined for ovalbumins purified from eight different sources all showed the following sequence: Gly-Asp/Gly-Ser*P*-Ile/Val/Met (Henderson et al., 1981).

Henderson et al. (1981) have noted that in all 16 phosphopeptides isolated from the 8 different ovalbumins the residue in position $n + 2$ is invariably glutamic acid. Moreover, all Ser-X-Glu or Ser-X-Ser*P* sequences in α- and β-caseins are always phosphorylated (Mercier et al., 1971; Ribadeau-Dumas et al., 1972). Thus, a negatively charged residue in position $n + 2$ may be a recognition point for a protein kinase. This view is supported by studies on genetic variants of casein; in one of these, the Glu-37 residue is replaced by a Lys, resulting in failure to phosphorylate Ser-35 (Grosclaude et al., 1972). It has been proposed that positively charged entities placed in position $n + 2$ or $n - 2$ are involved in the recognition by protein kinases (Williams, 1976). By contrast, the data summarized above are in support of a separate protein kinase specifically recognizing negative charges at position $n + 2$ (Meggio et al., 1981). In this context, it is noteworthy that phosphorylation sites within troponin I (Williams, 1976) and human fibrinogen (Taborsky, 1974) contain an acid residue at position $n + 2$ from a phosphorylated serine. It should be noted that ovalbumin contains three additional Ser-X-Glu peptides in its overall sequence that are not phosphorylated. It is possible that these sites are located on the inside of the molecule and cannot be reached by the protein kinase. Studies on these Ser-X-Glu-specific kinases from either mammary gland or chicken oviduct might aid in understanding the biological function of the structural phosphorylations of caseins and ovalbumins.

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References

Atkinson, P. H., Grey, A., Carver, J. P., Hakimi, J., & Ceccarini, C. (1981) *Biochemistry 20*, 3979–3986.
Bennick, A., McLaughlin, A. C., Grey, A. A., & Madapallimattam, G. (1981) *J. Biol. Chem. 256*, 4741–4746.
Blackburn, B. J., Lapper, R. D., & Smith, I. C. P. (1973) *J. Am. Chem. Soc. 95*, 2873–2878.
Bock, J. L., & Sheard, B. (1975) *Biochem. Biophys. Res. Commun. 66*, 24–30.
Brauer, M., & Sykes, B. D. (1981) *Biochemistry 20*, 2060–2064.
Breathnach, R., Mandel, J. L., & Chambon, P. (1977) *Nature (London) 270*, 314–319.
Chlebowski, J. F., Armitage, I. M., Tusa, P. P., & Coleman, J. E. (1976) *J. Biol. Chem. 251*, 1207–1216.
Flavin, M. (1954) *J. Biol. Chem. 210*, 771–784.
Greenberg, H., & Nachmansohn, D. (1965) *J. Biol. Chem. 240*, 1639–1646.
Grosclaude, F., Mahé, M. F., Mercier, J. C., & Ribadeau-Dumas, B. (1972) *Eur. J. Biochem. 26*, 328–337.
Henderson, J. Y., Moir, A. J. G., Fothergill, L. A., & Fothergill, J. E. (1981) *Eur. J. Biochem. 114*, 439–450.
Hofmeister, L. (1889) *Z. Physiol. Chem. 14*, 165.
Hunt, L. T., & Dayhoff, M. O. (1980) *Biochem. Biophys. Res. Commun. 95*, 864–871.
Ishihara, H., Takahashi, N., Ito, J., Takeuchi, E., & Tejima, S. (1981) *Biochim. Biophys. Acta 669*, 216–221.
Iwase, H., Kato, Y., & Hotta, K. (1981) *J. Biol. Chem. 256*, 5638–5642.
James, M. N. G., Delbaere, L. T. J., & Brayer, G. D. (1978) *Can. J. Biochem. 56*, 396–402.
Linderstrøm-Lang, K., & Ottesen, M. (1949) *C. R. Trav. Lab. Carlsberg 26*, 403.
Lingappa, V. R., Lingappa, J. R., & Blobel, G. (1979) *Nature (London) 281*, 117–121.
Long, W. F., & Williamson, F. B. (1980) *IRCS Med. Sci.: Libr. Compend. 8*, 808.
McCain, D. C., & Markley, J. L. (1980) *J. Am. Chem. Soc. 102*, 5559–5565.
McReynolds, L., O'Malley, B. W., Nisbet, A. D., Fothergill, J. E., Givol, D., Fields, S., Robertson, M., & Brownlee, G. G. (1978) *Nature (London) 273*, 723–728.
Meggio, F., Deana, A. D., & Pinna, L. A. (1981) *Biochim. Biophys. Acta 662*, 1–7.
Mercier, J. C., Grosclaude, F., & Ribadeau-Dumas, B. (1971) *Eur. J. Biochem. 23*, 41–51.
Milstein, C. P. (1968) *Biochem. J. 110*, 127–134.
Nanda, R. K., Ribeiro, A., Jardetzky, T. S., & Jardetzky, O. (1980) *J. Magn. Reson. 39*, 119–125.
Narita, K., Tsuchida, I., & Ogata, K. (1968) *Biochem. Biophys. Res. Commun. 33*, 430–435.
Neumann, H. (1968) *J. Biol. Chem. 243*, 4671–4676.

Nisbet, A. D., Saundry, R. M., Moir, A. J. G., Fothergill, L. A., & Fothergill, J. E. (1981) *Eur. J. Biochem. 115*, 335–345.
Osborne, J. B., & Campbell, G. F. (1900) *J. Am. Chem. Soc. 22*, 422.
Palmiter, R. D. (1975) *Cell (Cambridge, Mass.) 4*, 189–197.
Perlmann, G. E. (1955) *Adv. Protein Chem. 10*, 1–30.
Ribadeau-Dumas, B., Brignon, G., Grosclaude, F., & Mercier, J. C. (1972) *Eur. J. Biochem. 25*, 505–514.
Sarma, R. H., Mynott, R. J., Hruska, F. E., & Wood, D. J. (1973) *Can. J. Biochem. 51*, 1843–1851.
Swarup, G., Cohen, S., & Garbers, D. L. (1981) *J. Biol. Chem. 256*, 8197–8201.
Taborsky, G. (1974) *Adv. Protein Chem. 28*, 1–187.
Tai, T., Yamashita, K., Ito, S., & Kobata, A. (1977) *J. Biol. Chem. 252*, 6687–6693.
Vogel, H. J. (1982) Ph.D. Thesis, University of Alberta, Edmonton.
Vogel, H. J., Bridger, W. A., & Sykes, B. D. (1982) *Biochemistry 21*, 1126–1132.
Williams, R. E. (1976) *Science (Washington, D.C.) 192*, 473–474.
Yguerabide, J., Epstein, H. F., & Stryer, L. (1970) *J. Mol. Biol. 51*, 573–590.

Hydrogen-1, Carbon-13, and Phosphorus-31 Nuclear Magnetic Resonance Studies of the Dihydrofolate Reductase–Nicotinamide Adenine Dinucleotide Phosphate–Folate Complex: Characterization of Three Coexisting Conformational States†

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ABSTRACT: The *Lactobacillus casei* dihydrofolate reductase–folate–$NADP^+$ complex is shown by ^1H and ^{13}C NMR to exist in three interconverting conformational states, I, IIa, and IIb. The proportions of the three states, as estimated from the intensities of the three separate ^{13}C resonances observed in the complex containing [3-*carboxamido*-^{13}C]$NADP^+$, are pH dependent. State I predominates at low pH and states IIa and IIb predominate at high pH; the ratio IIa:IIb is pH independent. The pH dependence of the interconversion of states I and IIa + IIb can be explained by a model in which a group on the enzyme has a pK of <5 in state IIa + IIb and >7 in state I. ^1H, ^{13}C, and ^{31}P NMR has been used to characterize the structural differences between the three states of the complex. As judged by the ^1H and ^{13}C chemical shifts of the bound coenzyme, states I and IIa are similar to one another but quite different from state IIb. This difference appears to be a localized one, since only the nicotinamide 2 and 4 protons, nicotinamide 3-carboxamide ^{13}C, and pteridine 7 proton show differences in chemical shift between these states. These differences are, however, large—up to 1.4 ppm for ^1H and 2 ppm for ^{13}C. The remaining coenzyme protons, as well as the three ^{31}P nuclei, are unaffected. Studies of the C2 proton resonances of the seven histidine residues show that the ionizable group responsible for the interconversion of states I and IIa + IIb is not a histidine (although two histidines show slight differences in environment between states IIa and IIb); the possible identity of this ionizable group and the nature of the conformational differences between the states are discussed.

Dihydrofolate reductase, which is responsible for maintaining the cellular pools of tetrahydrofolate derivatives, is the target of the "antifolate" drugs trimethoprim and methotrexate, the latter being a close structural analogue of the substrate folate. There is considerable, though often indirect, evidence that ligand binding to the enzyme is accompanied by changes in protein conformation, which appear to be different for inhibitors and for substrates [see, e.g., Roberts et al. (1977), Blakely et al. (1978), and Feeney et al. (1980)]. These conformational changes have a considerable influence on the specificity of the enzyme (Birdsall et al., 1978, 1980a; Hyde et al., 1980a; Roberts, 1978) and most probably also underlie the cooperativity—both positive and negative—between coenzymes and substrates or inhibitors in their binding to the enzyme (Birdsall et al., 1980a,b, 1981a).

In addition to, and perhaps related to, these ligand-induced conformational changes, there is evidence that under some conditions the enzyme coexists as a mixture of two or more conformational states which interconvert only slowly. This appears to be the case in the absence of ligands for the enzyme from *Escherichia coli*, *Lactobacillus casei*, and *Streptococcus faecium* (Pattishall et al., 1976; Dunn et al., 1978; London et al., 1979; Cayley et al., 1981). Recently we have shown that the ternary complex of the *L. casei* enzyme with trimethoprim and $NADP^+$ exists as a mixture of comparable amounts of two conformational forms which bind the coenzyme differently and which interconvert at a rate of about 6 s^{-1} at 31 °C (Gronenborn et al., 1981a,b). Less direct evidence for two (or more) coexisting conformations has also been obtained for the ternary complex of the enzyme with coenzyme and the product analogue folinic acid (Birdsall et al., 1981a). We now report that the ternary complex containing enzyme, $NADP^+$, and the substrate folate exists in three states whose relative proportions are pH dependent. A preliminary report of part of this work has appeared (Birdsall et al., 1981b).

Materials and Methods

Dihydrofolate reductase was isolated from *L. casei* MTX/R as described by Dann et al. (1976). Its concentration was

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