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Structure of Hen Phosvitin: A ^{31}P NMR, ^{1}H NMR, and Laser Photochemically Induced Dynamic Nuclear Polarization ^{1}H NMR Study†

Hans J. Vogel*

ABSTRACT: ^{1}H nuclear magnetic resonance (NMR) studies of hen egg yolk phosvitin (M_r = 35000) have confirmed earlier suggestions that the protein has a very flexible and open structure and is not compactly folded. More than half of a total 220 residues in this unusual protein are phosphoserine residues. ^{31}P NMR pH titration studies have shown that all of these are in the dianionic form at physiological pH and that they are exposed to solvent and can be protonated and deprotonated. The pK_as determined for several groups of resonances that could be resolved in the ^{31}P NMR spectra were all close to those observed for phosphoserine standards. The low Hill coefficient (n = 0.70) calculated for these titration curves indicates that the phosphoryl moieties influence each others titration behavior. Subsequent laser photochemically induced dynamic nuclear polarization (photo-CIDNP) ^{1}H NMR experiments suggest that the single tyrosine and tryptophan residues are both exposed to solvent. Surprisingly, all 11 histidine residues appear to be inaccessible because only very small CIDNP effects were observed for these residues. Since experiments with mixtures of aromatic amino acids indicated that the presence of tryptophan or tyrosine decreases the CIDNP intensities observed for histidine residues, CIDNP spectra for phosvitin were compared with those obtained for mixtures of aromatic amino acids resembling the composition of this protein. This demonstrated that the histidine residues are partially inaccessible in the intact protein, suggesting that the histidine residues may be protonated and hydrogen bonded to the phosphoryl moieties. This concept was further supported by the results of the ^{1}H NMR titration studies that indicated that all 11 histidines have an unusually high pK_a = 7.45 and a low Hill coefficient for titration of n = 0.80.

Both major egg yolk proteins phosvitin and lipovitellin are derived from a common precursor called vitellogenin. This protein is synthesized in the livers of all egg-laying animals. Its synthesis is under hormonal control; administration of estrogen has been reported to increase the rate of transcription (Tata, 1976; Ryffel, 1978). Vitellogenin undergoes extensive posttranslational modifications: phosphorylation, glycosylation, and attachment of lipid moieties. After synthesis and processing in the liver, vitellogenin is transported through the blood to the ovary where it is cleaved to yield the egg yolk proteins before they are deposited in the yolk (Tata, 1976; Ryffel, 1978). Each vitellogenin molecule gives rise to two phosvitin molecules (Deeley et al., 1975). Highly purified preparations of phosvitin all show some heterogeneity (Taborsky, 1974). This property appears to be mainly related to the existence of different vitellogenin genes (Wiley & Wallace, 1981). The extent of the posttranslational modifications appears to be constant at least in preparations of hen phosvitin (Taborsky, 1974). Phosvitin (M_r = 35000) has a very unusual amino acid composition (see Table I). More than half of the residues are phosphoserines, accompanied by a fairly large number of acidic amino acids. The basic amino acids that are present are insufficient to balance all the negative charges and thus the protein is a polyelectrolyte. Parts of the protein have been sequenced (Belitz, 1965). These studies have led to the surprising discovery that blocks of usually six but sometimes up to eight SerP[1] residues may occur in one stretch. Such a stretch is usually terminated by a basic residue. Moreover, these blocks of SerP residues are evenly spread throughout the

Table I: Amino Acid Composition of Hen Phosvitin[a]

residue	no. of residues/mol of protein	residue	no. of residues/mol of protein
Gly	7	Thr	5
Ala	7	Cys	0
Val	3	Met	1
Leu	3	Asx	13
Ile	2	Glx	13
Pro	3	His	11
Phe	2	Lys	17
Tyr	1	Arg	11
Trp	1	(P)	(112)
Ser	120	total	220

[a] The data are the average of the six independent determinations described by Taborsky (1974).

† From the Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada. *Received May 24, 1982; revised manuscript received October 27, 1982.* Supported by the Killam Memorial Scholarship Committee and the Alberta Heritage Foundation for Medical Research.

* Address correspondence to the author at the Department of Physical Chemistry 2, Chemical Center, Lund, S220-07, Sweden.

[1] Abbreviations: DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; SerP, phosphoserine residue; ThrP, phosphothreonine residue; CIDNP, chemically induced dynamic nuclear polarization; FMN, flavin mononucleotide; Trp, tryptophan; Tyr, tyrosine; His, histidine; CD, circular dichroism; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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primary structure. This amino acid sequence provides for a protein with a very atypical structure. Its partial specific volume is only 0.54 mL/g (a "typical" protein value is 0.73 mL/g). Hydrodynamic measurements suggested an elongated shape whereas optical rotatory dispersion and circular dichroism studies indicated that no extensive α-helix or β pleated sheet structures are present at pH values where the phosphate residues are expected to be doubly negatively charged [for a review, see Taborsky (1974)].

The biological role of phosvitin is not well understood. It has been suggested that it may serve as an iron (Fe^{3+}) carrier in blood (as part of vitellogenin, Greengard et al., 1964; Ali & Ramsey, 1968) as well as in eggs (Taborsky, 1980).

Here ^1H NMR, ^{31}P NMR, and laser photo-CIDNP ^1H NMR studies of hen phosvitin are reported. These studies have confirmed that the protein has a flexible and unfolded structure that is ordered somewhat by an extensive network of rapidly exchanging charge and H-bond interactions. Earlier ^{31}P NMR studies had indicated that the majority of the phosphate residues are present in a SerP linkage and that the observed resonances shift with changes in pH (Ho et al., 1969). The phosphoserine residues have recently also been observed in crystalline lipovitellin–phosvitin complex from *Xenopus* (Banaszak & Seelig, 1982).

Experimental Procedures

Phosvitin was obtained from Sigma Chemical Co. and was used without further purification. The protein was judged to be at least 95% pure from its ^1H and ^{31}P NMR spectra. Protein for ^1H NMR and laser photo-CIDNP experiments was usually lyophilized once from D_2O to reduce the HDO resonance in the spectra. The samples were then dissolved in a buffer containing 50 mM KH_2PO_4, 0.05 mM EDTA, pH 7.0, and 99.8% D_2O (Bio-Rad). The pH (meter reading) was adjusted by adding small aliquots of 1 N NaOD and DCl solutions made up in 99.8% D_2O. Samples were centrifuged at 2000 rpm for 15 min to remove any insoluble material. The pH was measured on a Radiometer PHM62 standard pH meter equipped with a glass electrode. Samples for ^{31}P NMR were directly dissolved in a buffer containing 50 mM Tris-HCl, 50 mM maleic acid, 1 mM EDTA, and 35% D_2O at pH 8.3.

^{31}P NMR spectra (proton decoupled) were recorded on a Bruker HXS-270 spectrometer at a frequency of 109.3 MHz. Other conditions have been described elsewhere (Vogel & Bridger, 1982). Samples of 1.5 mL were placed in 10-mm round-bottom precision tubes (Wilmad) equipped with Teflon vortex plugs. All chemical shifts are referenced to 85% H_3PO_4. Upfield shifts are given a negative sign. ^1H NMR and laser photo-CIDNP ^1H NMR spectra were obtained on the same spectrometer at a frequency of 270 MHz. ^1H NMR chemical shifts are referenced to DSS, with a positive sign given to downfield shifts. Samples (1 mL) were placed in 10-mm flat-bottom precision tubes fitted with vortex plugs. The pulse angle applied was approximately 90°, and the time between individual pulses for the ^1H NMR experiments was 0.5 s. All spectra were obtained with homonuclear continuous wave decoupling to suppress the HDO resonance. Laser photo-CIDNP experiments were performed essentially as described by Hincke et al. (1981) with the following modifications. All samples contained 5 μL of a 50 mM FMN (Sigma Chemical Co.) stock solution in 100% D_2O. Alternating light and dark spectra were obtained and stored and added on the disk (Kaptein, 1978, 1982). Two different sequences were used, but in both, a laser pulse with an intensity of 3.0 W was used (Spectra Physics Model 164 argon ion laser operating in the multiline mode). Sequence 1 employed a 1.0-s laser pulse before the acquisition of the spectrum and a 20-s delay between individual light pulses; in sequence 2, a 0.8-s laser pulse was used that was repeated every 16 s. Results with either sequence were virtually identical. A series of control experiments with mixtures of aromatic amino acids and the proteins ovalbumin and lysozyme[2] had shown that under these conditions close to optimal CIDNP enhancements were obtained while minimizing bleaching of the dye and modification of amino acids by possible photooxidation [see also McCord et al. (1981)]. Since these last two effects influence the intensities observed, fresh samples were used for each laser photo-CIDNP experiment that involved measuring the intensities. Usually 16 scans (for protein) or 8 scans (for amino acids) were collected; under these conditions the reproducability was better than 10%. All NMR experiments were performed at a temperature of 28 ± 1 °C.

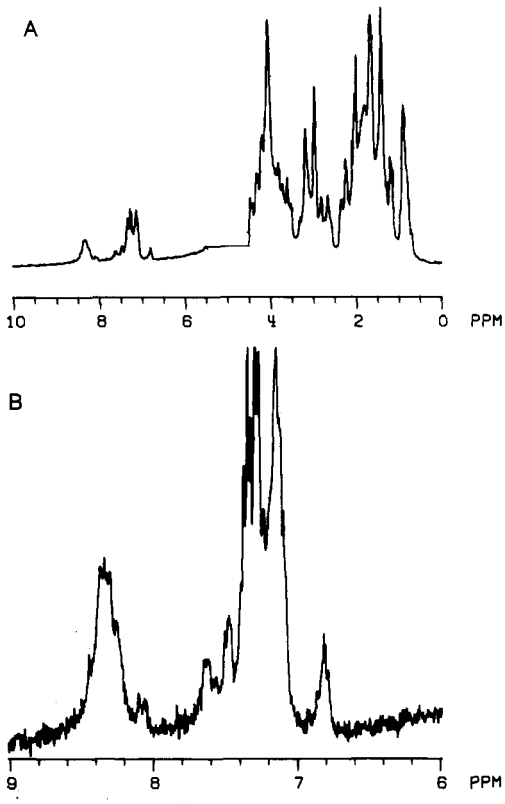

FIGURE 1: (A) 270-MHz ^1H NMR spectrum of hen phosvitin at pH 7.0. 1000 acquisitions were taken of a 0.5 mM phosvitin sample; the delay between individual pulses was 1.4 s. An additional line broadening of 1 Hz was introduced by the computer digital filtering. (B) Aromatic region of the 270-MHz ^1H NMR spectrum of phosvitin. No additional line broadening.

Results and Discussion

^1H NMR Studies. Figure 1A shows a ^1H NMR spectrum obtained for a sample of phosvitin. It shows that all the resonances in the aliphatic region of the spectrum are remarkably well resolved for a protein of this size (M_r = 35000). The spectrum bears more resemblance to that of a mixture of amino acids (Wüthrich, 1976) than to spectra published for proteins with similar molecular weight. The sharp resonance at 0.9 ppm represents all the methyl groups in the protein. The resonance at 4.1 ppm is remarkably high in comparison to spectra obtained for other proteins and reflects the SerP methylene group. Since homonuclear decoupling was used to suppress the residual HDO line in this spectrum, the

[2] H. J. Vogel and B. D. Sykes, unpublished results.

intensities are likely to be effected by cross-relaxation phenomena and thus unfortunately cannot be used for direct estimation of the amino acid composition.

Multiplication of the free induction decays with a sinebell results in line narrowing for sharp resonances and removes broad resonances from the spectrum (Gassner et al., 1978). When this technique was applied to phosvitin, all resonances sharpened in the ^1H NMR spectrum. In contrast, similar manipulations of free induction decays collected for ovalbumin samples virtually removed all resonances from the spectrum (data not shown); ovalbumin is compactly folded (Taborsky, 1974) and is thus expected to give rise to broad resonances. The above suggests that no broad resonances exist for phosvitin, indicating the protein to be very flexible. This notion found further support in T_1 inversion recovery measurements performed again on both ovalbumin and phosvitin. In compactly folded large proteins, cross-relaxation (or spin diffusion) will effectively eliminate differences between T_1 values for the different amino acid residues (Kalk & Berendsen, 1976; Sykes et al., 1978). Indeed, all resonances in the compactly folded ovalbumin had identical T_1 values. However, all resonances in the ^1H NMR spectrum of phosvitin had different T_1 values, again suggesting that the protein is unfolded (data not shown). No unexchangeable NH residues are found in the spectra shown in Figure 1B. Identical results were obtained when the protein was dissolved directly prior to the acquisition of the spectrum. This gives additional support to the idea that the protein is unfolded and flexible, since all backbone NH residues can rapidly exchange with solvent D_2O (Wüthrich, 1976).

Assignment of Aromatic Resonances in the ^1H NMR Spectrum. Figure 1B shows the aromatic region of the 270-MHz ^1H NMR spectrum of phosvitin. The resonance centered around 6.79 ppm gives rise to a large emission in a laser photo-CIDNP spectrum (see Figure 4) and thus represents the 3 and 5 protons of the carboxy-terminal tyrosine residue (Taborsky, 1974). The corresponding tyrosine 2 and 6 protons appear at 7.07 ppm. They can be clearly resolved at high or low pH when the His H_4 protons (in Figure 1B centered around 7.14 ppm) are shifted. The resonances observed for the tyrosine ring protons shifted upfield at pH >10, indicating that the tyrosine phenolic residue has a normal pK_a. CIDNP spectra as shown in Figure 4 (cf. further below) indicate that the tryptophan H_2 and H_6 resonances appear at 7.22 ppm and the H_4 resonance appears at 7.62 ppm. The resolved resonance observed at 7.48 ppm probably corresponds to the tryptophan H_7 proton. The broad resonance observed centered around 8.4 ppm is a composite of narrower resonances of the H_2 protons of all 11 histidine residues as discerned by its titration behavior. Also, the little flank at 8.1 ppm shifts with the characteristics of a histidine H_2 resonance. The set of very sharp resonances centered around 7.35 ppm represents the tryptophan H_5 proton and the ortho, meta, and para protons of the two phenylalanine residues. The resonances observed for the phenylalanine protons are very narrow in comparison to the resonance of Tyr H_3 and H_5 and the Trp H_4 and H_7 resonances, suggesting that the former are free to rotate and do not interact with the protein. Neither phenylalanine nor tryptophan is present in any of the partial sequences reported for phosvitin [for a review, see Taborsky (1974)]. Thus, the assignment of the differential mobility to certain segments of the protein awaits the completion of the primary structure.

^{31}P NMR pH Titration Studies. Figure 2 shows a series of ^{31}P NMR spectra obtained for a sample of hen phosvitin at different pH values. The spectra represent all the SerP

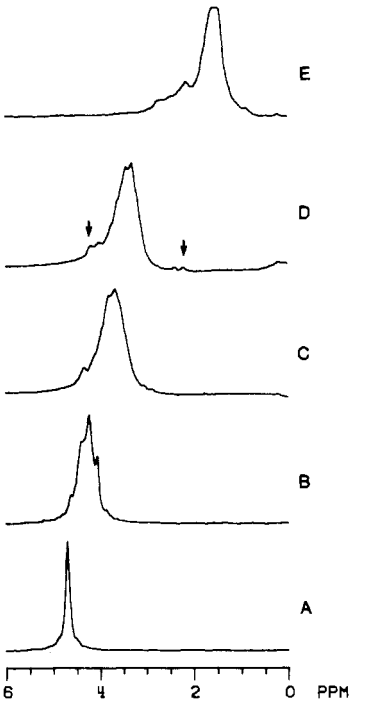

FIGURE 2: ^{31}P NMR spectra of 1 mM phosvitin at several pH values: (A) pH 11.4; (B) pH 9.9; (C) pH 7.5; (D) pH 6.8; (E) pH 5.4. All spectra are plotted on the same scale, and 200 acquisitions were collected for each spectrum.

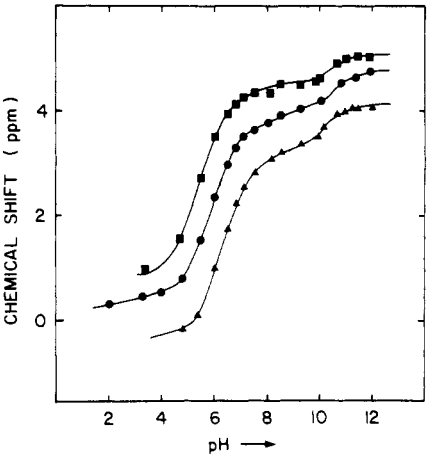

FIGURE 3: ^{31}P NMR pH titration curves obtained for some resolved groups of resonances in the spectra of phosvitin: (■) left resonance; (●) major resonance; (▲) right resonance.

residues present in the phosvitin molecule; the broad peak arises from overlapping resonances for all these residues. All resonances shift with pH in a way characteristic of phosphomonoesters (Vogel & Bridger, 1983), indicating that all SerP residues are exposed to solvent. It is also readily apparent that not all phosphoryl moieties are equivalent and that many have slightly different environments and/or pH titration behavior. The arrows in Figure 2D indicate the resonances whose titration behavior has been determined, in addition to that of the major resonance. These data are shown in Figure 3. It is noteworthy that changes in shift are observed at higher pH values (compare panels A and B of Figure 2). Normally, no changes in chemical shifts are observed in this pH range for the titration of standard phosphomonoester solutions (Vogel & Bridger, 1983). The titration data in Figure 3 clearly illustrate that these inflections are observed for all resonances. As outlined before, phosvitin contains much fewer basic residues than SerP moieties, but apparently all of these SerP

residues sense the presence of the basic residues leading to the high pH inflection observed for all pH titration curves. This can be interpreted as evidence that the salt linkages between the positively charged basic residues and the negatively charged SerP residues are in fast exchange between all phosphoryl moieties.[3]

The larger inflection in the titration curves observed at lower pH values corresponds to the usual titration for the conversion of the dianionic to the monoanionic species. The pK_as and Hill coefficients (n) were calculated for the lower inflections of the titration curves with a nonlinear least-squares analysis program (Markley, 1975). They were pK_a = 6.17, 5.79, and 5.35 and n = 0.71, 0.70, and 0.76, respectively. For a SerP standard, these values are 5.75 and 0.92, respectively. Obviously, some of the protein-bound residues have pK_as different from that of the standard. However, it is noteworthy that the major peak representing at least 80% of all the residues has a pK_a very similar to that of a standard. A low Hill coefficient ($n \ll 1$) indicates the presence of other charged groups titrating in the same pK_a range (Markley, 1975). Thus the values of n = 0.70 suggest that all SerP residues have others close by that affect their titration behavior. Similar results for the rat incisor dentine phosphoprotein have been explained by assuming an extensive hydrogen-bonding network between the ionic side chains (Cookson et al., 1980).

Of particular interest is the chemical shift position and titration behavior of the resonance indicated by the right arrow in Figure 2, since these bear more resemblance to a threonine phosphate than a serine phosphate residue (Vogel & Bridger, 1983). Careful amino acid analyses have in fact suggested the presence of one ThrP residue in the protein (Allerton & Perlmann, 1965). Since the intensity of this resonance suggests that it represents one residue, it is tempting to suggest that the observed resonance corresponds to this residue. Unfortunately, however, studies of proton-coupled spectra have not allowed differentiation between a doublet proton–phosphorus splitting as would be expected for a ThrP or a triplet splitting as would be expected for a SerP residue (Ho et al., 1969).

Laser Photo-CIDNP ^1H NMR Spectra. This novel technique has been introduced recently to facilitate the detection and further study of protein-bound exposed aromatic residues (Kaptein, 1978). In general, a flavin dye is added to a ^1H NMR sample, and the dye is excited to its triplet state in the probe of the NMR spectrometer by brief laser irradiation. The photoexcited dye can form a radical pair with accessible Trp, Tyr, and His residues, resulting in spin polarization accompanied by enhancement of NMR signals. These can be most readily detected by subtracting a dark from a light spectrum, giving as a net result the CIDNP difference spectrum (Kaptein, 1978, 1982; Berliner & Kaptein, 1981). Since the earlier measurements indicated that phosvitin has an unfolded structure, it could be reasoned that the flavin dye should be able to approach all His, Trp, and Tyr residues present and this in turn should give rise to very intense CIDNP signals for most resonances in the aromatic part of the ^1H NMR spectrum. Figure 4 shows the results obtained for a 1 mM phosvitin sample. It shows the light (Figure 4B) and dark (Figure 4A) ^1H NMR spectrum. Several enhanced resonances can be readily detected by a comparison of the aromatic regions of both these spectra. Figure 4C shows the light minus

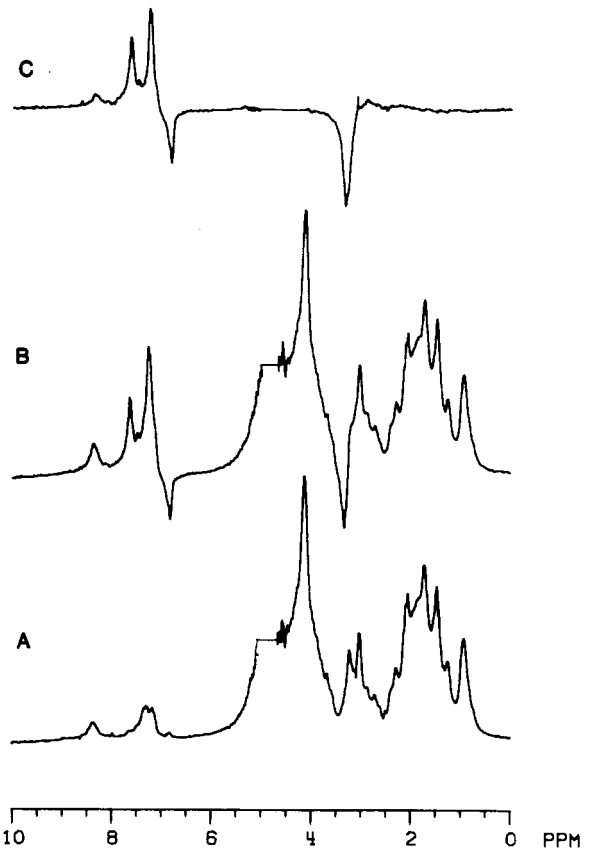

FIGURE 4: Laser photo-CIDNP ^1H NMR spectra of 1 mM phosvitin (pH 6.9): (A) dark spectrum; (B) light spectrum; (C) CIDNP difference spectrum obtained by subtracting the light and dark spectra. 16 acquisitions were collected for these spectra.

dark CIDNP difference spectrum. The emission line at 6.80 ppm originates from the tyrosine $H_{3,5}$ protons. The strong absorptions represent the enhancements for the Trp $H_{2,6}$ (7.22 ppm) and Trp H_4 (7.61 ppm). The strong emission at 3.30 ppm represents the β-methylene protons of the Trp residue.[4] Surprisingly, the His H_2 resonance at 8.35 ppm is only weakly enhanced. (For a comparison to standard CIDNP spectra, see Kaptein (1978).] The enhancements for the tyrosine and tryptophan protons are all at least a factor of 15 (compare panels A and C of Figure 4). This number compares favorably to those observed for standard amino acids (Kaptein, 1978; Hincke et al., 1981; McCord et al., 1981). However, the virtual absence of any enhancement for the His protons may suggest that all 11 histidine residues are inaccessible to the dye.

In a further experiment the effect of the number of acquisitions on the intensities of the CIDNP effect was investigated. The effect did not increase linearly with the total number of acquisitions as is normally observed with other spectroscopic techniques. This deviation arises from a gradual bleaching of the dye and the fact that some modification of aromatic amino acids occurs in the course of the experiment as could be demonstrated by comparing normal ^1H NMR spectra recorded immediately before and after the CIDNP experiment (data not shown). The same dependence was found for the strongly polarized Tyr and Trp and the weakly enhanced His H_2 residues, confirming that none of the aromatic

[3] Another possibility is that this high pH inflection represents the release of a last proton from a phosphoserine block. Although such behavior is unusual for pH titrations of phosphorus compounds (Vogel & Bridger, 1983), it may occur on compounds with a high charge density (Zuiderweg et al., 1979).

[4] A small enhancement can be detected at 7.46 ppm. This represents the Trp H_7 resonance. In free amino acids this proton is usually not enhanced; however, cross-relaxation phenomena may give rise to its appearance in protein-bound Trp residues (de Kanter & Kaptein, 1979).

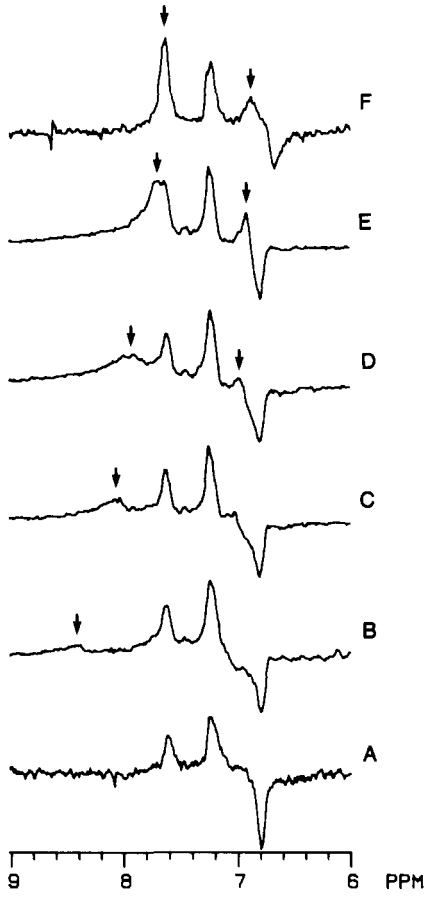

FIGURE 5: CIDNP spectra obtained for phosvitin at different pH values. The arrows indicate the resonances observed for the histidine H_2 (left) and H_4 (right) resonances, respectively: (A) pH 2.95; (B) pH 5.75; (C) pH 8.05; (D) pH 8.70; (E) pH 10.0; (F) pH 10.6.

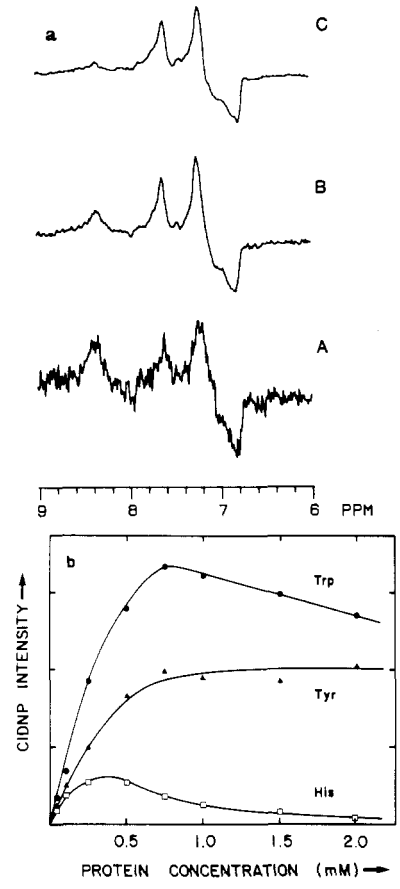

FIGURE 6: (a) CIDNP spectra of phosvitin obtained at different protein concentrations (pH 7.0): (A) 0.05 mM; (B) 0.25 mM; (C) 1.0 mM. All spectra are plotted on the same scale. (b) CIDNP intensities (in arbitrary units) measured for the phosvitin, tryptophan, tyrosine, and histidine H_2 resonances as a function of protein concentration (pH 7.0).

residues are selectively modified by photooxidation and thus rendered insensitive to further observation. Hence, the absence of a strong CIDNP effect for the His residues is not caused by selective photooxidation of these residues.

pH Dependence of CIDNP Spectra. Since all phosphoryl moieties of phosvitin can be readily protonated to monoanionic species, changes in pH could be expected to bring about large differences in accessibility of the His residues. This possibility is further addressed by the set of spectra depicted in Figure 5. The arrows indicate the CIDNP effects observed for the His H_2 (left) and His H_4 resonances (right). It appears that the His residues become somewhat accessible at higher pH, but the effect is not as much as expected for a total of 11 histidine residues. The deprotonated histidine amino acid itself is known to give rise to more intense CIDNP effects than in the protonated form (Kaptein, 1982) and thus the slight increase in the enhancement observed for the protein is more likely to be due to this effect than to a pH-dependent change in accessibility. At the lowest pH values tested, no His CIDNP effects are observed although virtually all phosphate residues should be protonated to a monoanionic species. Again, this does not mean that all His residues are inaccessible at low pH, since similar behavior is seen for the free amino acid (Kaptein, 1982). Note that the tyrosine emission has shifted and decreased in intensity at the highest pH. Both effects are caused by the titration of the phenolic hydrogen.

Hydrogen atom abstraction is suggested to be a necessary step in the formation of a radical pair, resulting in CIDNP observed for Tyr and His residues (Kaptein, 1978, 1982). Thus deprotonation of tyrosine residues results in a decrease of the CIDNP effect. Experiments with *O*-methyltyrosine showed that this compound did not give rise to CIDNP effects. Furthermore, experiments with N^1- and N^3-methylhistidine showed that, although CIDNP effects were observed at low pH, these compounds at high pH did not support formation of a radical pair (data not shown; Kaptein, 1978, 1982). These data support the idea that hydrogen abstraction is a requirement for CIDNP effects with either His or Tyr. In contrast, N^1-methyltryptophan gives CIDNP spectra very similar to those of the normal amino acid, suggesting that in this case electron transfer is the primary step to observe CIDNP effects (McCord et al., 1981; Kaptein 1978, 1982).

Concentration Dependence of Phosvitin CIDNP Spectra. In the course of our studies, we noticed unexpected changes in the relative intensities of the resonances observed in the CIDNP spectra. This is illustrated by the spectra presented in Figure 6a. The histidine H_2 resonance appears most prominent[5] in the spectra obtained at the lowest protein concentration and seems to decrease at higher protein concentrations. A complete concentration dependence is shown in Figure 6b. Clearly, the intensities observed for the resonances do not increase linearly with the concentration. Trp reaches a maximum at 0.75 mM and decreases from then on; tyrosine levels of at about 0.75 mM, and the histidine intensity is always very low and decreases beyond a concentration of 0.35 mM. The behavior of Trp was paralleled by similar effects seen for a standard[2] (McCord et al., 1981). However, the CIDNP

[5] The His H_4 resonance is also more prominent, but this one overlaps with the Trp $H_{2,6}$ resonance.

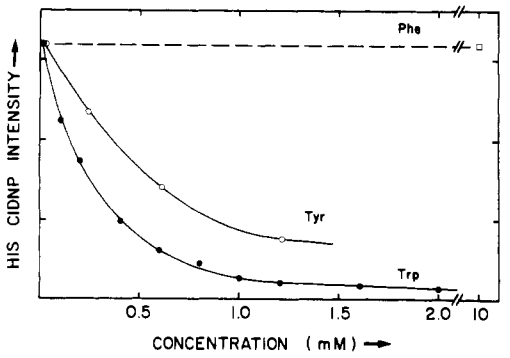

FIGURE 7: CIDNP intensities (in arbitrary units) measured for the His H_2 resonances of a 2 mM histidine solution (at pH 7.0) in the presence of varying amounts of other aromatic amino acids.

effect, determined for a His standard measured under identical conditions to those described for the experiment shown in Figure 6b, increased linearly to a concentration of about 3 mM.[2] Thus the behavior of the His in the protein appeared anomalous. A possible explanation for this phenomenon is that Trp and Tyr can preferentially interact with the dye. Thus the increase in Trp and Tyr residues, which of course occurs concomitantly with the increase in phosvitin concentration, would then effectively reduce the CIDNP effect observed for the His residue. This possibility is further explored in the experiments described in the following section.

CIDNP Experiments with Mixtures of Amino Acids. Figure 7 shows the data obtained for experiments with samples that all contained a constant amount of histidine (2 mM) and varying amounts of tryptophan, tyrosine, or phenylalanine. The intensities measured for the His H_2 resonance in the CIDNP spectra are obviously a function of the presence of the amino acids Trp and Tyr. Both of these give themselves strong CIDNP effects as well. Addition of Phe, which does not give rise to a CIDNP spectrum itself, does not result in a decrease. These effects of Tyr and Trp could be of competitive nature, but both residues are known to be capable of forming complexes with the dye as well (Draper & Ingraham, 1970; Isenberg & Szent-Györgyi, 1959; Fleischman & Tollin, 1965). Strict competition for available dye molecules would not explain the decrease in CIDNP effects observed for Trp at higher Trp concentrations (see Figure 6b; McCord et al., 1981); thus it seems likely that the preference of the dye for Trp and Tyr over His (see Figure 7) may originate in their complexation with the dye. This would also provide an explanation for the stronger effects of Trp on the intensity of the His than those of Tyr (see Figure 7); the former is known to bind the dye more tightly (Draper & Ingraham, 1970).

These experiments provide a possible explanation for the failure to observe intense CIDNP effects for the protein-bound His residues. Moreover, they also suggested the following control experiment: if phosvitin is entirely unfolded and all His residues are exposed, its CIDNP spectrum should resemble that of a mixture of aromatic amino acids of identical composition.[6] Comparison of spectra obtained for such a mixture and phosvitin showed that the intensity measured in the amino acid mixture for the His H_2 and H_4 resonances exceeds the ones measured in the protein, whereas the intensities for the Trp and Tyr appeared about equal. Thus some feature of the protein must be responsible for the low intensities measured

[6] In the case of phosvitin, such a comparison is possible due to the unfolded and flexible nature of the protein. However, not for all proteins is such practice allowed, since slower protein motions may affect the CIDNP intensities [see Kaptein (1982)].

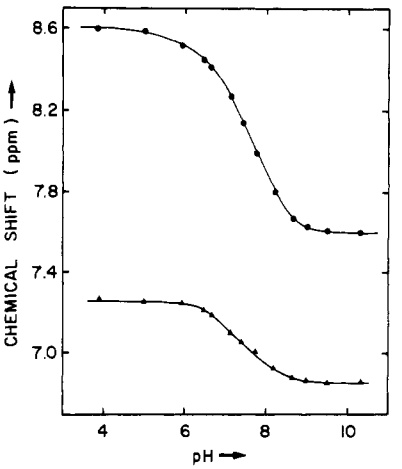

FIGURE 8: ^1H NMR pH titration curves measured for the histidine H_2 (●) and H_4 (▲) resonances of phosvitin.

for the His. In the foregoing, it was suggested that inaccessibility may play a role. However, as discussed in the previous section, hydrogen abstraction is a requirement for the observation of CIDNP effects of histidine residues. Recently, it was suggested that exposed surface histidine residues of ribonuclease did not give rise to CIDNP effects because of their involvement in hydrogen bonds (Bolscher et al., 1979). Thus it seems likely that the histidine residues of phosvitin are hydrogen bonded to the phosphoryl moieties. If such hydrogen bonding does indeed occur, one would predict that these residues would have an unusual titration behavior: they are all expected to have a high pK_a and a low Hill coefficient (Markley, 1975).

^1H NMR pH Titration Study of Phosvitin. Figure 8 shows the ^1H NMR titration curves obtained for the His H_2 and His H_4 residues, respectively. In the course of these titrations, some heterogeneity for the 11 histidine residues became apparent. However, it was not possible to resolve any of the lines clearly, and the majority behaved like the broad resonance shown in Figure 1B. Significantly, all His resonances shifted with pH, indicating their exposure to solvent. The pK_as and Hill coefficients for the titration curves were calculated by nonlinear least-squares analysis. They were $pK_a = 7.51$ and 7.42 and $n = 0.79$ and 0.82 for the H_2 and H_4 resonances, respectively. The average pK_a of 7.47 differs significantly from the value of 6.9 determined for the tripeptide Gly-His-Gly. Moreover, the n value of 0.80 suggests the presence of other groups in the vicinity of the protons that titrate in a similar pH range (Markley, 1975). Thus these data are in support of the idea that all histidine residues of phosvitin are hydrogen bonded to the phosphoryl moieties of this protein.

Conclusions

These studies have shown that phosvitin is a highly unfolded protein. All serine phosphate residues, all backbone amide protons, and the single tyrosine and tryptophan residues are all exposed to solvent. Although the 11 histidine residues are only slightly accessible to the dye in a CIDNP experiment, all 11 can be titrated, indicating their exposure to solvent. The protein is not only unfolded but is also very flexible as was suggested by the narrow resonances observed in the ^1H NMR and ^{31}P NMR spectra and by the T_1 inversion recovery measurements. Some ordering is brought into all of this by the hydrogen bonding between the histidines and the SerP residues as implied by the CIDNP experiments and by the salt linkages formed between the basic amino acids and the phosphoryl moieties as inferred from the ^{31}P NMR studies.

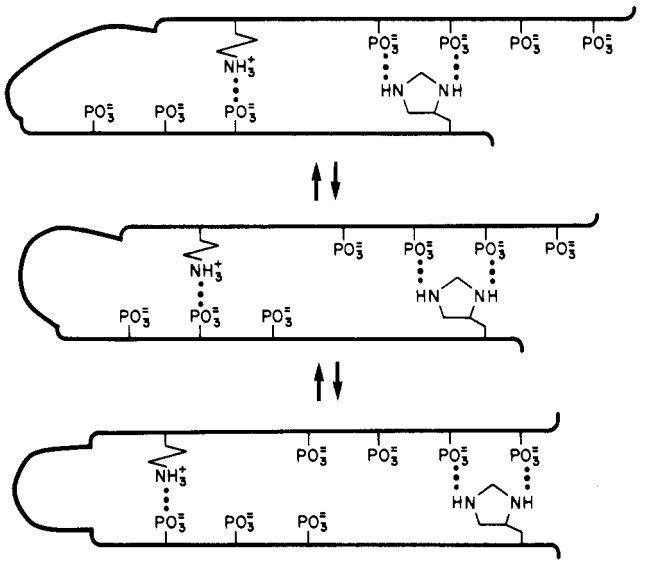

FIGURE 9: Schematic model indicating the rapidly exchanging interactions between the basic amino acids, histidine residues, and phosphoryl moieties of phosvitin.

Such linkages occur only intramolecularly since no changes in the intrinsic viscosity were detected for samples with protein concentrations between 0.2 and 2.0 mM (data not shown). A schematic representation consistent with these observations is presented in Figure 9. It is suggested that all hydrogen-bond and salt linkages can exchange rapidly between all the phosphoryl moieties within one stretch of phosphoserine residues. This exchange allows for the flexibility observed. It is of interest that the partial sequence determinations have shown that a large portion of the Arg, Lys, and His residues are present in between stretches of phosphoserine residues (Belitz, 1965). Such an arrangement fits very well with the model presented in Figure 9. The carboxy-terminal tyrosine is not involved in hydrogen bonding; it would not have shown a strong CIDNP spectrum, analogous to the histidines, if it were involved in the H bonding. The Trp residue may be involved in hydrogen bonding since this would not interfere with the electron transfer that results in CIDNP for this residue (McCord et al., 1981; Kaptein, 1982). Most likely, bound metal ion can also exchange very rapidly between all different phosphoryl moieties. Recently, similar models have been proposed for the binding of metal ions to the polyphosphate side chain of ATP and its analogous (Vogel & Bridger, 1982) and for the binding of metal ions to another polyelectrolyte protein, the rat incisor dentine phosphoprotein (Cookson et al., 1980).

It should further be mentioned here that the results of the laser photo-CIDNP experiments indicate that the interpretation of such spectra is not as straightforward as one would hope. On the basis of the results of the experiment shown in Figure 7, anomalous intensity distributions can be expected for studies of all proteins. These data indicate that the intensity of a resonance is not a direct measure of its exposure nor can changes in the intensities be directly interpreted in terms of changes in exposure or hydrogen bonding.

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Registry No. Tyr, 60-18-4; Trp, 73-22-3; His, 71-00-1; L-phosphoserine, 407-41-0.

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