Conformational Transition of the Phosphoprotein Phosvitin. Random Conformation → β Structure*

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ABSTRACT: The effect of various organic solvents on the conformation of the phosphoprotein phosvitin was investigated utilizing optical rotatory dispersion and circular dichroism. The data suggest that phosvitin, which in the pH range of 3.0–5.0 has an essentially unordered conformation, undergoes a coil to β transition when either ethylene glycol, methanol, ethanol, 2-propanol, *tert*-butyl alcohol, or dioxane is added to its aqueous solvent. The optical rotatory dispersion patterns of phosvitin at pH 3.0–5.0 have two negative minima at 207 and 233 nm, and a positive peak at 194 nm which on addition of organic solvents change to the Cotton effects of the β form with a 230–232-nm trough and a 202–204-nm peak, respectively. Similarly, the circular dichroism spectra characterized in aqueous solution by a negative band at 197 nm in the presence of the organic solvent display a positive peak centered at 192–195 nm and a negative band at 216 nm. These transitions from unordered to β structure are reversible. At pH 2.0, phosvitin has a β structure. Addition of the various solvents increases the reduced mean residue rotation, $[m']_{202}$, and the reduced mean residue ellipticity, $[\theta']_{195}$, while the position of the negative Cotton effect and ellipticity band remain unchanged. In the pH range of 6.0 to 10.0 the optical rotatory dispersion patterns and circular dichroism spectra of the protein are characteristic of an unordered conformation which is affected by the organic solvent only inasmuch as $[m']$ and $[\theta']$ increase. The degree of transition to the β form not only depends on the nature of the organic solvent added but also on the concentration of the organic constituent. These studies implicate the importance of environmental factors in the control of the polypeptide backbone conformation. In the case of a protein with polyelectrolyte characteristics such as phosvitin the conformational characteristics are influenced to a certain extent by the dielectric constant of the medium.

In a previous study we have described the optical rotatory dispersion curves and circular dichroism spectra of the phosphoprotein phosvitin. From the results of this investigation which comprised a wide range of pH and ionic strength we concluded that at alkaline pH phosvitin has an "unordered" conformation, whereas at pH 3.0–4.6 small amounts of α-helical regions and/or β structure are present within an otherwise unordered structure (Grizzuti and Perlmann, 1970). While this work was in progress it was shown by Taborsky that the conformation of phosvitin changes to a β-type structure when the ionization of the phosphomonoester groups of this highly negatively charged protein is suppressed on lowering the pH from 3.4 to 1.0 (Taborsky, 1968). This observation and the important finding that organic solvents such as dioxane and methanol were able to convert the optical rotatory dispersion patterns of "disordered silk" into the β form (Iizuka and Yang, 1966) prompted us to investigate the effect of various organic solvents upon the conformation of phosvitin. We will show in the present work that in organic solvents the transition from an unordered conformation to β structure occurs already at an apparent pH of 4.0–5.0 and is to a certain degree dependent on the dielectric properties of the solvents and solvent mixtures used.

Materials and Methods

Two phosvitin preparations with a nitrogen content of 12.96 and 13.50% and a phosphorus content of 11.40 and 12.28% were isolated from fresh hens' eggs according to the procedure of Joubert and Cook (1958).

All chemicals were commercial analytical reagents and were not further purified. The various organic solvents used were either "Spectrograde" or "Chromatoquality" and were purchased from Matheson Coleman and Bell (ethylene glycol, dioxane, and *tert*-butyl alcohol), Merck (2-propanol), and Eastman Organic Chemicals (methanol).

The aqueous constituents of the solvents used were 0.01 N HCl, distilled water, Na phosphate buffer, pH 7.7, Γ/2 0.1, and NaOH–glycine buffer, pH 10.3, Γ/2 0.01.

In the experiments at various ionic strengths, NaCl solutions of appropriate concentrations were used to attain a final NaCl concentration in the organic solvent of 0.005 and 0.01; at higher salt concentrations phosvitin precipitated. On adding a salt solution to the organic solvent the highest concentration of methanol that could be used without precipitating the protein was 50%, v/v.

Since phosvitin is not soluble in the organic solvents used, fresh stock solutions were prepared for each set of experiments by dissolving the protein in water or in the respective buffer. The organic solvent was then added to attain the desired concentration of the organic constituent.

In experiments designed to test the dependence of the optical rotatory dispersion and circular dichroism on the protein concentration the stock solutions of phosvitin were diluted to the appropriate protein content with the respective solvent.

In the reversibility experiments removal of the organic solvents was achieved by dialysis of the solutions at 4° for 24 hr against large amounts of distilled water or buffer with frequent changes of the dialysate.

Slight opalescence appeared sometimes in the HCl-containing solvent mixtures; in those instances the solutions

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TABLE I: Position of Cotton Effects and Dichroic Bands of L-Polypeptides and of Phosvitin in Various Organic Solvents.

Conformation	Optical Rotatory Dispersion (nm)			Dichroic Bands (nm)		
	Trough	Crossover	Peak	Negative	Crossover	Positive
L-Polypeptides, unordered	204–205	198	190	199–200		
Phosvitin, pH 3.0–5.5	207	199	192	197	189	Nr[a]
Phosvitin–50% methanol (v/v)	232	222	202	216	204	192
Phosvitin–50% ethylene glycol	230	218	Nr	215	203	Nr
Phosvitin–50% 2-propanol	232	219	199	215	203	Nr
Phosvitin–50% dioxane	231	220	Nr	215	Nr	Nr
Phosvitin–50% *tert*-butyl alcohol	230	220	200	215	200	Nr
Phosvitin–50% ethanol	230	220	200	215	201	Nr
Phosvitin, pH 2.0	232	221	205	216	208	195
L-Polypeptides, β form	229–230	221	204	218	208	197

[a] Nr, not recorded because of high solvent absorption.

were filtered through Millipore filters. In some cases soft, jelly-like, highly opalescent products were obtained by allowing the solution to stand overnight. In spite of the turbidity, the optical rotatory dispersion patterns and circular dichroism spectra were reproducible within a few degrees of those obtained on the previous day with clear solutions. This further corroborates that the Cary 60 spectropolarimeter is relatively insensitive to scattered light, as has also been reported by other investigators (Lenard and Singer, 1966; Wallach and Zahler, 1966; Steim and Fleischer, 1967; Hammes and Schullery, 1968, 1970).

For the optical rotatory dispersion and circular dichroism measurements the stock solutions of phosvitin were 0.25–0.5% in the wavelength range of 600–300 nm, and 0.01–0.1% in the far-ultraviolet region.

All the protein concentrations except those in the glycine buffer are based on nitrogen analysis by the Pregl micro-Kjeldahl method with a mercuric catalyst (Hiller *et al.*, 1948). In the glycine buffer the protein concentration was obtained by phosphorus determinations as described elsewhere (Grizzuti and Perlmann, 1970). The nitrogen and phosphorus factors based on separate nitrogen, phosphorus, moisture, and ash determinations of the individual phosvitin preparations were used for conversion into dry weight.

The pH of all solutions was measured with the Radiometer pH meter Model 4, calibrated with the standard buffers recommended by Bates (1954). Addition of organic solvents increased the apparent pH by about 1.0 to 1.5 pH units as compared with the aqueous solvent and the values given represent the apparent pH. Changes of the optical rotatory dispersion and circular dichroism due to this variation in pH were ignored since they are very small compared with the initial solvent effect (Tooney and Fasman, 1968). The pH values thus reported in the organic solvents containing 10 to 90% of the organic constituent are average values.

Optical rotatory dispersion and circular dichroism were performed at 25° on a Cary recording spectropolarimeter, equipped with the 6001 circular dichroism attachment in 10-mm, 1-mm, and 0.5-mm path-length cells.

The results of the optical rotatory dispersion in the wavelength range of 600 to 300 nm are expressed as specific optical rotation, $[\alpha]$, whereas below 300 nm they are given as mean residue rotation, $[m]$, where the mean residue molecular weight was taken as 161 (Allerton and Perlmann, 1965). The circular dichroism is recorded in terms of ellipticity in degrees and in analogy to $[m]_\lambda$ expressed as $[\theta]_\lambda$. All our results were corrected for the variations of the index of refraction of the solvents in the manner reported elsewhere (Grizzuti and Perlmann, 1970) and are reported as $[m']_\lambda$[1] and $[\theta']_\lambda$. The variation of the refractive index with wavelength and solvent concentration were calculated from available sources (Tooney and Fasman, 1968; Handbook of Chemistry and Physics, 1958; Handbook of Biochemistry, 1968).

The dielectric constants for each mixture of buffer or water with 2-propanol, *tert*-butyl alcohol, ethylene glycol, and methanol were computed with the aid of the values given by Åkerlöf (1932) and in the International Critical Tables (1929). Dioxane and ethanol were assumed to follow the same pattern.

Results

Optical Rotatory Dispersion and Circular Dichroism in the Wavelength Range 260–190 nm. EFFECT OF ORGANIC SOLVENTS. As has been reported elsewhere, the optical rotatory dispersion curves of phosvitin at pH 3.6–5.0 display two minima at 207 nm and at 232 nm with residue rotations $[m']_{207}$ −5200 and $[m']_{232}$ −3415, a maximum at 192 nm with $[m']_{192}$ 9500, and a crossover point at 198 nm (Perlmann and Allerton, 1966; Timasheff *et al.*, 1967; Grizzuti and Perlmann, 1970). The optical rotatory dispersion of phosvitin in aqueous solution, however, changes drastically when 30% (v/v) or higher concentrations of either ethylene glycol, methanol, or ethanol are present in the solutions. Similarly, the circular dichroism spectra of an aqueous solution of phosvitin, characterized by a negative band at 197 nm with $[\theta']_{197}$ −20,000 and a shoulder at 220 nm, is also altered upon addition of an organic solvent (Timasheff *et al.*, 1967). As shown in Table I, in the presence of organic constituents the positions of the minima, crossover points, and maxima are shifted from those typical of the unordered conformation to bands characteristic of β structures.

Figure 1 illustrates the optical rotatory dispersion patterns and circular dichroism spectra of phosvitin in an aqueous

[1] Abbreviations used are: $[m']$, reduced mean residue rotation; $[\theta']$, reduced mean residue ellipticity; $[\alpha]$, specific optical rotation; λ_c, optical rotatory dispersion constant.

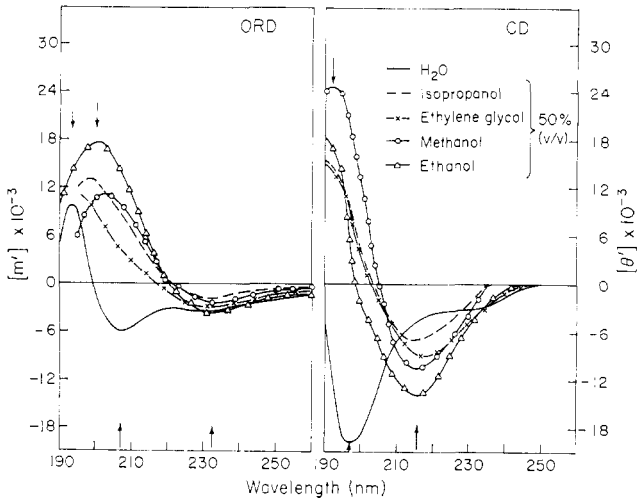

FIGURE 1: Optical rotatory dispersion and circular dichroism of phosvitin in water and various organic solvents of apparent pH 4.6.

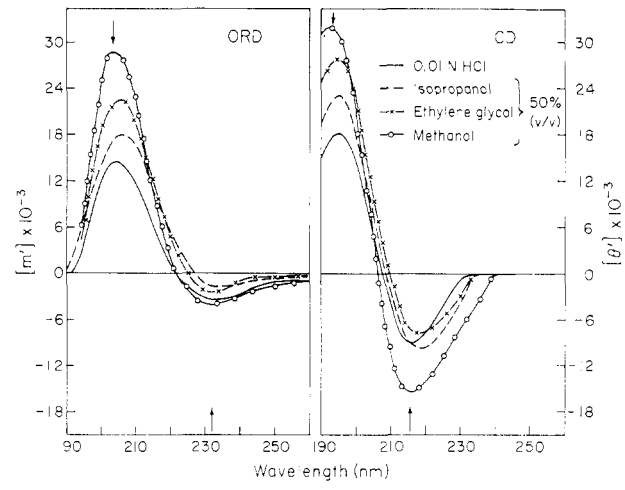

FIGURE 3: Optical rotatory dispersion and circular dichroism of phosvitin in water and various organic solvents of apparent pH 2.4.

solution of pH 4.6 and the effect of added ethylene glycol, methanol, and ethanol (50%, v/v). From this figure it is apparent that the values recorded for $[m']$ and $[\theta']$ differ with the various solvents. Thus at 202 nm $[m']_{202}$ is 11,700 and 17,000 for methanol and ethanol, respectively. On the other hand, the trough at 230 nm changes less drastically. Furthermore, while in the optical rotatory dispersion patterns the maximum remained within the same wavelength range, *i.e.*, 199–202 nm, regardless of the organic solvent used and, as will be discussed below, is only shifted after a critical concentration of the organic constituent is reached, in the circular dichroism spectra the deep minimum at 197 nm with $[\theta']_{197}$ −20,000 has disappeared and has been replaced by a peak at 192 nm in methanol. The positive bands of phosvitin in the other solvents used must occur below 190 nm and therefore could not be recorded. The negative band of the circular dichroism spectra is at 215–217 nm. Furthermore, there is a quantitative difference in the circular dichroism spectra in the various solvents used. Thus, at the apparent pH of 4.6 the ellipticity, $[\theta']_{215}$, in 50% (v/v) 2-propanol and dioxane is −8582 ± 1500, while in ethanol and ethylene glycol $[\theta']_{215}$ is −13,000 ± 3200 and $[\theta']_{215}$ is −9300 ± 2000, respectively. It should further be noted that on removal of the organic solvent constituent by dialysis against distilled water, the optical rotatory dispersion patterns and circular dichroism spectra are identical with those of the aqueous phosvitin solutions given in Figure 1. This indicates reversibility of the conformational changes observed.

Effect of Ionic Strength. As mentioned in the preceding section, the transition from unordered to β structure is affected by the presence of various organic solvents. Furthermore, addition of NaCl to a phosvitin solution in methanol affects the optical rotatory dispersion patterns and circular dichroism spectra of the protein. If the NaCl concentration exceeds 0.01 N, 30% methanol produces a heavy precipitate in a 0.5% phosvitin solution. Therefore, our measurements were confined to NaCl concentrations in the range 0–0.01 N and to methanol–NaCl mixtures not exceeding 50% methanol. The interesting feature of the data reported in Figure 2 is the increase of $[m']_{205}$ and $[\theta']_{197}$ on addition of salt to the organic solvent, suggesting that the presence of NaCl enhances the formation of β structure. Thus, in the solvent with 50% methanol $[m']_{202}$ increases from 11,000 to 28,000. Similarly, $[\theta']_{192}$ is 34,000 and $[\theta']_{217}$ is −16,000 in the presence of 0.01 N NaCl while in the absence of salt $[\theta']_{192}$ is 25,000 and $[\theta']_{217}$ is −11,000, respectively. It should further be noted that a 0.5% phosvitin solution in 50% methanol–0.01 N NaCl (v/v) upon standing forms a soft opalescent gel with trapped-in bubbles that has thixotropic properties in that it liquefies by shaking and re-forms on standing. The optical rotatory dispersion and circular dichroism properties, however, are not affected.

Effect of pH. 1. pH 2.0. It has been reported by Taborsky that phosvitin will undergo a transition from an unordered conformation to β structure on lowering the pH of the protein solution to 1.8 and thereby repressing the ionization of the charged phosphate groups (Taborsky, 1968). We have confirmed these results and have established that presence of an organic solvent enhances the formation of β structure (Figure 3). A comparison of the optical rotatory dispersion patterns and circular dichroism spectra at the apparent pH of 2.4 of Figure 3 with those of pH 4.6 given in Figure 1 reveal the same effectiveness of the organic solvent to promote the formation of the β form. As reflected in changes of $[m']_{207}$ and $[\theta']_{195}$, the following order of effectiveness was found: aqueous solution < 2-propanol < ethylene glycol < methanol. In a

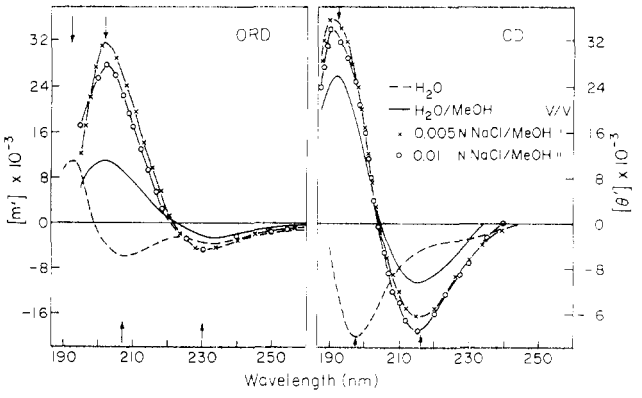

FIGURE 2: Optical rotatory dispersion and circular dichroism of phosvitin in water, 50% aqueous methanol, and in 50% NaCl–methanol mixture (v/v) of apparent pH 4.6.

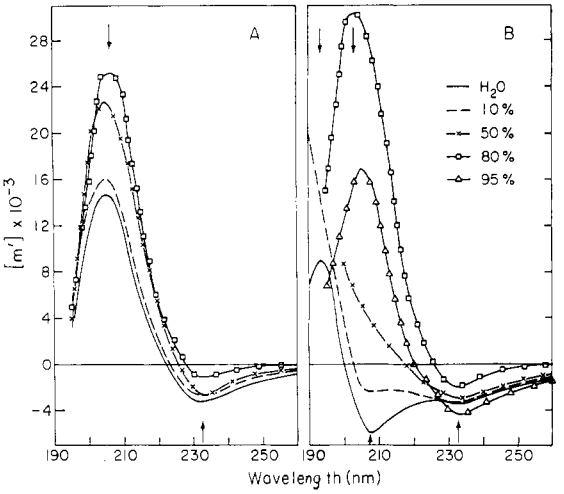

FIGURE 4: Optical rotatory dispersion of phosvitin in aqueous solution and ethylene glycol of different concentrations: (A) apparent pH 2.4; (B) apparent pH 4.6.

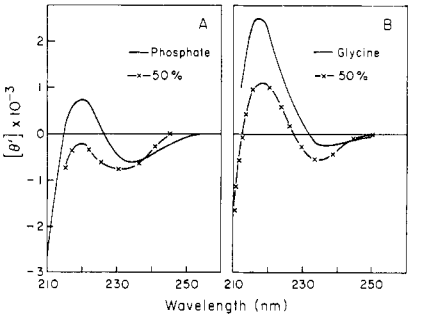

FIGURE 5: Circular dichroism of phosvitin in water and aqueous ethylene glycol (50% v/v) at apparent pH 7.8 (A) and 9.6 (B).

solvent containing 50% (v/v) organic constituent, $[m']_{207}$ increases from 15,800 to 18,000, 22,000, and 28,000 and $[\theta']_{195}$ from 18,000 to 23,000, 27,000, and 32,000, respectively. The trough in the optical rotatory dispersion patterns at 232 nm and the negative band at 215 nm are less affected.

Having demonstrated that addition of an organic solvent at pH 4.6 will result in a transition from an unordered conformation to a β structure and that at pH 2.0 the presence of the organic constituent will enhance the β form, it was of interest to investigate the effect of solvent concentration on the optical rotatory dispersion patterns. Figure 4 illustrates the results obtained in ethylene glycol where the concentration was altered from 10 to 95%. It is of particular interest that $[m']_{207}$ in 95% ethylene glycol is lower than in 80%, an observation also made with other organic solvents.

2. NEUTRAL AND ALKALINE pH. In the pH range 6.0–11.0 the optical rotatory dispersion patterns and circular dichroism spectra are those of a protein with an unordered conformation. This is readily understood since phosvitin in this pH range is highly charged and the electrostatic repulsion of the charged side chains maintains the protein in an extended conformation and thus, on addition of organic solvents, prevents transition to β structure. However, the values of $[m']_{205}$ and $[\theta']_{198}$ change upon addition of the organic solvents; $[m']$ and $[\theta']$ become less levorotatory. This is exemplified by a series of experiments in which the concentration of ethylene glycol was varied from 0 to 70%, (v/v) (Table II). Similarly, the addition of ethylene glycol also changes the weak dichroic bands observed in the wavelength range 210–250 nm (Grizzuti and Perlmann, 1970). In Figure 5 is illustrated the effect of 50% ethylene glycol upon the fine structure region. Thus, the ellipticity $[\theta']_{220}$ decreases from 750 to −250 and from 2500 to 1000 at pH 7.8 and 9.6, respectively.

Interpretation of Results. In this study of the optical rotatory dispersion and circular dichroism of phosvitin in the far ultraviolet reported in the preceding sections we have established that a transition of the unordered to β structure occurs on transferring the protein from a pure aqueous solution to an organic solvent. In all solvents tested phosvitin was already partially in the β form in 30% organic solutions while in 65–70% at the same pH (pH 4.6, apparent) the transition was complete. These findings are substantiated by the Cotton effect curves and dichroism spectra of Figures 1 and 2. A simple explanation for our results can be found from the changes of the degree of dissociation of the phosphate groups of the phosphoserine residues of phosvitin brought about by altering the dielectric properties of the solvent from that of pure water to that of the organic constituent.

We have already mentioned that phosvitin at pH 3.0–5.0 has a negative Cotton effect at 207 nm. On addition of an organic solvent the negative residue rotation, $[m']_{207}$, becomes positive until $[m']_{207}$ passes a maximum and decreases slightly at 90–95% (v/v) of the solvent mixture. To ascertain the relation of the unordered to β form transition on solvent composition and on the dielectric properties, we followed $[m']_{207}$ in ethylene glycol, methanol, and ethanol (Figure 6). Transition curves are obtained with midpoints corresponding to a dielectric constant of 57.6, 56.2, and 50.2 for ethylene glycol, methanol, and ethanol, respectively. These transition midpoints correspond to solvent composition within the range 50–60% of the organic constituent. Thus the existence of a certain relationship of the dielectric properties of the solvent to the conformational change of the protein emerges.

Optical Rotatory Dispersion of Phosvitin in the Wavelength Range 600–350 nm. As to date no information in the visible range is available on the optical rotatory dispersion of proteins and polypeptides present in β structure. Therefore, we wish to record our findings on phosvitin in the various organic solvents. We shall show that marked deviations from a simple optical rotatory dispersion occur during the transition from the unordered conformation to β structure.

TABLE II: Dependence of the Optical Rotatory Dispersion and Circular Dichroism of Phosvitin in the Far Ultraviolet on Ethylene Glycol Concentration and pH.

Ethylene Glycol, % (v/v)	pH 7.8		pH 9.7	
	$-[m']_{205}$	$-[\theta']_{197}$	$-[m']_{205}$	$-[\theta']_{197}$
0	14,300	24,800	19,500	27,800
10	13,700	12,000	18,900	25,200
30	13,400	20,500	14,100	23,000
50	8,800	17,900	11,200	21,800
70	8,100	19,200	9,100	16,500
80	Nr[a]	Nr	7,400	14,600

[a] Nr, not recorded because of high solvent absorption.

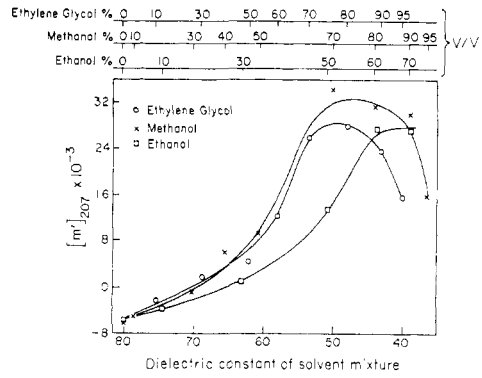

FIGURE 6: Unordered → β structure transition of phosvitin in ethylene glycol, methanol, and ethanol at pH 4.6. Dependence on dielectric properties and composition of solvents.

TABLE III: Dependence of the Specific Optical Rotation, $[\alpha]_{366}$, of Phosvitin on Solvent Composition.

Solvent Composition, % (v/v)	Solvent				
	Ethylene Glycol	Methanol	Ethanol	*tert*-Butyl Alcohol	Dioxane
0	−204	−204	−204	−204	−204
10	−161	−176	−207	−179	−196
30	−134	−148			−165
50	−96	+49	−18	−120	+49
60	−11	+85			+39
70	+114	+62			
80	+95	+15	+78	+30	
90	+57				

Solvent Effects on the Dispersion. In the spectral range 600–350 nm, phosvitin in water is levorotatory. The specific optical rotation $[\alpha]_{366}$ −204° and the rotatory dispersion can be represented by the one-term Drude equation with the optical rotatory dispersion constant λ_c 230 nm (Jirgensons, 1958; Perlmann and Allerton, 1966; Grizzuti and Perlmann, 1970). The effect on $[\alpha]_{366}$ of the organic solvents of various compositions is illustrated with the aid of Table III. It is apparent that in analogy to the results in the far ultraviolet $[\alpha]_{366}$ becomes less levorotatory on increasing the concentration of the organic solvent constituent. $[\alpha]_{366}$ is dextrorotatory when the protein is present in the β form as ascertained from the positions of the optical rotatory dispersion Cotton effects and circular dichroism bands in the far ultraviolet (Figure 1).

In Figure 7 are given dispersion curves in ethylene glycol at concentrations of 0–95% (v/v). The dispersion constant, λ_c, obtained from the plot of $[\alpha]$ *vs.* $[\alpha] \times \lambda^2$ (Yang and Doty, 1957) increases from 230 in water to 243 nm in 50% ethylene glycol. At higher ethylene glycol concentrations the dispersion is more complex and can not be described by the one-term Drude equation.[2] Using the plot of $[\alpha]$ *vs.* $[\alpha] \times \lambda^2$, a curvature is observed similar to that reported for poly-γ-benzyl-L-glutamate in chloroform (Yang and Doty, 1957).[3] Although the dispersion of phosvitin in methanol and ethanol is similar to that in ethylene glycol, λ_c in dioxane and *tert*-butyl alcohol decreases from 230 to 220 nm at low concentrations of these solvents.

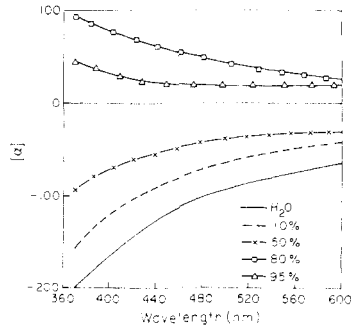

FIGURE 7: Rotatory dispersion of phosvitin in water and in ethylene glycol of different concentrations at apparent pH 4.0.

It should also be pointed out that, while phosvitin is soluble in ethylene glycol and methanol up to 95%, the limiting concentration of dioxane which does not produce formation of a precipitate from a 0.5% phosvitin solution is 60%.

The changes observed for the rotatory dispersion at neutral and alkaline pH in the presence of an organic solvent follow the pattern described above.

Time Dependence. The effect of time on the transition from unordered to β structure was tested at solvent concentrations which produced the maximal changes, *i.e.*, 80% glycol, 70% methanol, and 50% methanol in 0.01 N NaCl. Usually, an invariant rotation was recorded after 15 min. However, the changes that occur during the initial period do not exceed 15° at 366 nm, indicating that the conformational transition occurs relatively fast.

In conclusion it should be stated that our results on the optical rotatory dispersion of phosvitin in organic solvents in the wavelength range 600–350 nm follow a similar pattern to that described by Yang and Doty (1957) for poly-γ-benzyl-L-glutamate. Although these investigators are dealing with an α-helix → coil transition and β aggregates, whereas in the case of phosvitin the conformational changes observed are those of a transition from an unordered to a β structure, it is not unlikely that in both cases these changes in the optical rotatory dispersion are in general representative of the formation of an ordered structure.

Discussion

The principal conclusion reached in the foregoing presentation that phosvitin in organic solvents is present in β structure is based upon the observations that the optical rotatory dispersion and circular dichroism of the protein in various organic solvent mixtures resemble those of polypeptides in β form and of silk fibroin (Iizuka and Yang, 1966). This simple hypothesis seems justified for the interpretation of our results for the following reasons: In the organic solvent mixtures, the dielectric constant, ε 78, of pure water

[2] Attempts to fit the dispersion data using the Moffitt equation with a λ_0 212 nm in several cases did not give a straight line. In cases when a straight line was obtained b_0 varied between +113 and −200 (*cf.* Jirgensons, 1965, 1966).

[3] With one or two exceptions, the b_0 of the Moffitt equation for the β form given in the literature appears to be close to zero (Moffitt and Yang, 1956).

is lowered considerably. Since it is well known that the apparent pK' of acids increases in organic solvents (*cf.* Iizuka and Yang, 1966),[4] a similar trend should also appear in the charged side groups of a protein. Hence, it is not surprising that on transferring phosvitin, a protein with a content of 56% phosphoserine, to an organic solvent the dissociation of the phosphate groups is suppressed or shifted to a more alkaline pH, thus reducing the electrostatic repulsion between the charged side chains. Hence, a transition from an unordered conformation to a β structure can take place. This is reminiscent of the transition that occurs on lowering the pH of a phosvitin solution below 2.0 (Taborsky, 1968).

Although lowering the activity of the water molecules by altering the dielectric constant, ϵ, is essential to provide a less polar medium, a certain correlation between the effectiveness of the organic solvent in producing this conformational transition and the dielectric properties of the solvent was observed. However, it can be stated that in ethylene glycol, methanol, and ethanol, where the dielectric constants of the pure solvents vary from 38, 33, to 24, respectively, the midpoint of the transition corresponds to an ϵ_0 of 58, 56, and 50, or to a solvent composition of 50–60% (v/v).[5] The maximal amount of β structure under the conditions studied in our laboratory is obtained with 70–80% of aqueous ethylene glycol or methanol. Presence of 0.005 N NaCl in 50% (v/v) methanol enhances the formation of β structure. This is readily understood since interaction of the phosphate groups with Na^+ may reduce the polarity of the charged groups.

We turn finally to a comparison of our results with observations on the β structure of poly-L-serine. In water this polymer is characterized by optical rotatory dispersion patterns with a trough at 233 nm and a positive Cotton effect at 210 nm (Davidson *et al.*, 1966; Fasman and Potter, 1967; Tooney and Fasman, 1968). The circular dichroism spectra with maxima at 197 nm and minima at 220 nm are also characteristic of the β form (Quadrifoglio and Urry, 1968). Furthermore, it was reported by these investigators that the rate of interconversion from random coil to β structure is enhanced by the presence in the solvent of large amounts of trifluoromethanol, methanol, dioxane, or acetonitrile. The higher the content of organic solvents the faster the enhancement of the β type circular dichroism curve (Quadrifoglio and Urry, 1968). Therefore, in view of the high content of phosphoserine in phosvitin, it is not surprising that this protein will undergo such a conformational transition if the side-chain charges are repressed.

The location of the Cotton effects in the optical rotatory dispersion spectra of the β form of phosvitin puts this protein in the form I-β of the Fasman and Potter classification (1967). However, it is not possible to state which particular β form is obtained, either parallel or antiparallel pleated sheets. The question of how much β structure is present in phosvitin also arose. We attempted to fit the circular dichroism curves in 70% methanol and 80% ethylene glycol at pH 4.6 (apparent) with the computed circular dichroism spectra for poly-L-lysine calculated by Greenfield and Fasman (1969). In both cases, by the $[\theta']_{217}$ criteria, phosvitin, under these conditions, would contain 80% β and 20% random coil. However, if one looks at the peak in the 195–192-nm region less clear-cut answers are obtained and the possibility of the presence of some α helix cannot be ruled out entirely.[6]

Similar calculations based on the circular dichroism spectrum of phosvitin in 50% (v/v) aqueous methanol at pH 2.4 (apparent) gives 100% β conformation. The increase of the peak at 202–205 nm upon the addition of organic solvent suggests that also at pH values below 2.0 some other conformation may be present in aqueous solutions of phosvitin.

Acknowledgment

We would like to dedicate this paper to Dr. Lewis G. Longsworth whose retirement on July 1, 1970, coincided with the completion of this work. We should like to thank him for encouragement in our work in the past and we shall miss his valuable advice in the future.

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[4] The apparent pK' of acetic acid increased from 4.6 in water to 8.1 in 50% dioxane (*cf.* Iizuka and Yang, 1966).

[5] Due to solubility problems which did not permit exploration to the full range of solvent concentration, the results given in Figure 6 were limited to these alcoholic solutions.

[6] Dr. W. Traub of the Weizmann Institute of Science, using X-ray diffraction on our phosvitin preparation, has been able to demonstrate that on orienting of phosvitin at pH 4.6 by freeze-drying, the X-ray patterns of the solid protein are characteristic of the pleated-sheet β structure. We wish to thank Dr. Traub for having communicated to us his results prior to publication (W. Traub, to be published) (*cf.* also Taborsky, 1970).

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Effects of pH on the Rate of Acid Denaturation of Horse Oxy-, Deoxy-, and Other Ferrohemoglobins*

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ABSTRACT: The pH profiles for acid denaturation of oxyhemoglobin (O_2Hb) and deoxyhemoglobin (Hb^0) have been measured by working at 0° and low pH to minimize oxidation (with O_2Hb) and by working under strictly anaerobic conditions (with Hb^0). Under certain conditions, O_2Hb is kinetically even more stable than carbonylhemoglobin (COHb). Plots of the log rate constant against pH give parallel straight lines with slopes of 2.4 at pH 2.4–3.6. Hb^0 is denatured about 100 times faster than O_2Hb. To test an earlier hypothesis that large differences in stability between Hb^0 and O_2Hb are due to differences in the electronic configuration of the heme iron, we measured the rates of denaturation of nitric oxide hemoglobin (NOHb), nitrosobenzene hemoglobin (C_6H_5NOHb), and hemoglobin complexes of various alkyl isocyanides (RNCHb), all of which are diamagnetic. In comparison with O_2Hb and COHb, they have a low kinetic stability – about the same as Hb^0 and ferrihemoglobin (Hb^+), which are paramagnetic. It is suggested that the relatively large size of the ligands may counteract any stability conferred on the protein by formation of covalent bonds. Spectroscopic data for one of the isocyanide complexes (ethyl, EtNCHb) indicates that although denaturation (unfolding), as determined by changes in the ultraviolet spectrum, is a one-step process, formation of the final product is a multistep process, possibly involving formation of multiligated species. Optical rotatory dispersion spectra show that the pH of half-denaturation for EtNCHb at equilibrium is 4.2. Perhaps more striking than the differences in kinetic stability is the fact that only two slopes are observed for the various pH profiles: 3.3 for $N_3^-Hb^+$, COHb, NOHb, and *i*-PrHb; 2.4 for all other complexes for which data are available. Thus, the difference in slopes appears to be unrelated to either the electronic configuration or to the charge of the heme iron. Stability at any given pH and slope of the pH profiles are also uncorrelated.

Extensive investigations on the kinetics and thermodynamics of acid denaturation of hemoglobin have previously been carried out largely with ferrihemoglobin (Hb^+) because the ferrohemoglobins undergo oxidation to Hb^+ in denaturation experiments which are not painstakingly anaerobic (Steinhardt *et al.*, 1966). When low-spin complexes of Hb^+, namely with N_3^- and CN^-, were used, it was found that they are kinetically stabilized toward acid denaturation (Steinhardt *et al.*, 1963; Molday and Steinhardt, 1969). CNS^- and F^-, ligands which form high-spin complexes, have only a small stabilizing effect. More recently, extensive anaerobic investigations of ferrohemoglobin as carbonylhemoglobin (COHb) have been carried out (Steinhardt *et al.*, 1966; Geddes and Steinhardt, 1968; Allis and Steinhardt, 1970). It has been found that COHb is about 100 times more stable than Hb^+, and is about as stable as $N_3^-Hb^+$ and CN^-Hb^+. In contrast to the case of Hb^+ where breaking of the heme imidazole bond and denaturation are simultaneous events, with COHb the heme bond persists in the denatured protein above pH 3 (Allis and Steinhardt, 1970). Below pH 3 the heme is removed at a rate slower than the denaturation; hence, the two reactions can be separated. This separation is facilitated by the fact that separate isosbestic points exist in the ultraviolet spectrum for each reaction.

Little work has been done on the stability of other ferrohemoglobins, especially those which are most important physiologically, *i.e.*, oxyhemoglobin (O_2Hb) and deoxyhemoglobin (Hb^0). In this paper we report the effect of pH on the rate of the acid denaturation of O_2Hb and Hb^0. The purpose of this more extended investigation was an attempt to confirm the hypothesis of Steinhardt *et al.* (1963) that the stability of hemoglobins toward acid denaturation may depend on either (a) the formation of a low-spin (covalent) bond between the heme iron and imidazole nitrogen; or (b) the absence of a net positive charge on the heme group; or both. We also measured the pH profiles of acid denaturation for nitric oxide ferri- and ferrohemoglobins ($NOHb^+$ and NOHb, respectively) and those of various alkyl isocyanide complexes of ferrohemo-

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