Further Studies on the Binding of Divalent Cations to the Phosphoglycoprotein Phosvitin†

Kärt Grizzuti* and Gertrude E. Perlmann‡

ABSTRACT: Dialysis equilibrium measurements at 25° indicate that, at pH 6.8 and at a concentration of 1.0×10^{-3} *M* $MnCl_2$ or $CoCl_2$, phosvitin binds 113 Mn^{2+} and 120 Co^{2+}. The binding is cooperative at low cation concentrations. The number of Mg^{2+}, Ca^{2+}, Mn^{2+}, and Co^{2+} bound is not affected by temperatures of up to 60°; however, the cooperativity is enhanced. Optical rotatory dispersion and circular dichroism studies indicate that a conformational change occurs on binding of Mn^{2+} and Co^{2+} which parallels the one produced by Ca^{2+} and reported elsewhere [Grizzuti, K., and Perlmann, G. E. (1973), *Biochemistry 12,* 4399]. The conformational changes induced by Mg^{2+} and Mn^{2+} follow different paths. Upon binding of Mn^{2+} and Co^{2+} the intrinsic viscosity, $[\eta]$, of phosvitin decreases from about 0.5 to 0.03 dl/g, while Mg^{2+} and Ca^{2+} decrease $[\eta]$ to 0.048 dl/g. The ultraviolet absorption spectrum of phosvitin is altered upon binding of Ca^{2+}, Mn^{2+}, and Co^{2+}, but not upon binding of Mg^{2+}; an increase of the temperature to 60° has no further effect on the spectra.

In a previous study we examined the binding of Ca^{2+} and Mg^{2+} to the phosphoglycoprotein phosvitin (Grizzuti and Perlmann, 1973). From the results of that investigation we concluded that at pH 6.5 and 25° phosvitin binds 160 Ca^{2+} and 140 Mg^{2+}, respectively, which is in fair agreement with the analytical data derived from amino acid analyses (Allerton and Perlmann, 1965). Furthermore, it was suggested that the binding of these two ions is of electrostatic nature, that it involves weak and independent binding sites of similar affinity, and is accompanied by a conformational change.

Seeking ion specificity, we extended this study to the binding of Mn^{2+} and Co^{2+} to phosvitin. The first section of the paper contains the results of an investigation on the mode and extent of binding of these two divalent cations at pH 6.8 and 25°. We shall show that in contrast to Ca^{2+} and Mg^{2+} at low concentrations of Mn^{2+} and Co^{2+} the binding is of cooperative nature. In addition we shall discuss the effect of temperature on the binding of the divalent ions thus far studied.

The second section of the paper contains the effect of the binding on the viscosity and optical properties, such as optical rotatory dispersion (ORD), circular dichroism (CD), and ultraviolet absorption in the wavelength range of 330–240 nm.

Materials and Methods

Materials. Two different phosvitin preparations with a nitrogen content of 13.2 and 12.8% and phosphorus of 11.4 and 12.4% were isolated from fresh hen's eggs according to the procedure of Joubert and Cook (1958). The metal content was 3–4 Mg^{2+} and/or Ca^{2+} per mole of phosvitin. No Mn^{2+} or Co^{2+} was found.

Eriochrome Black T was purchased from Matheson Coleman and Bell, Erio SE from Eastman Organic Chemicals; 4-hydroxy-3-nitroso-1-naphthalenesulfonic acid was purchased from J. T. Baker Chemical Co. All other chemicals were reagent grade.

Methods. The binding experiments were performed at 25, 37, and 60°. At 25° the dialysis equilibration was done as described in a previous article (Grizzuti and Perlmann, 1973) using the 9 ml of protein–90 ml of dialysate method. To perform equilibrium dialysis at 37 and 60° we placed the tubes containing 90 ml of the solvent (a mixture of the divalent ion in sodium cacodylate buffer pH 6.8 and $\Gamma/2$ = 0.02) in a Haake circulating water bath (Type NBS) to equilibrate to the desired temperature. The dialyzing bags containing 9 ml of the phosvitin solution containing 2.2 mg/ml were then placed into the large tubes. Dialysis was carried out with gentle stirring, using a TRI-R Model MS-7 submersible stirring unit (Rockville Center, New York, N.Y.) for 22–24 hr to assure equilibration.

Specific viscosities were measured at 25 ± 0.02° in an Ostwald-type viscometer with a flow time for the sodium cacodylate buffer (pH 6.8 and $\Gamma/2$ = 0.02) of 63 sec. Viscosities of each phosvitin solution were obtained at several protein concentrations in the range of 0.03–0.6% and were used to compute the intrinsic viscosities. The diffusates after equilibration had been attained were used for the dilution of the protein stock solutions.

ORD and CD measurements were carried out at 25° in a Cary 60 recording spectropolarimeter equipped with a 6001 circular dichroism attachment as described previously (Grizzuti and Perlmann, 1969). ORD and CD titrations were performed with solutions containing 0.4 and 0.2 mg of phosvitin/ml, respectively, and 0.05 *M* $MgCl_2$ and $MnCl_2$. The phosvitin solutions were placed in quartz cells and the cation solutions were added stepwise. After each 0.25 μM increment in cation concentration the optical rotation and the ellipticity were followed until an invariant recording was obtained which was then used for computations.

Ultraviolet absorption spectra were recorded on a Zeiss PMQ-II spectrophotometer equipped with a thermostatable cell holder using stoppered quartz cells of 3.0-ml capacity with 1-cm path length and a phosvitin solution of 0.72 mg/ml. The spectra of phosvitin solutions containing Ca^{2+},

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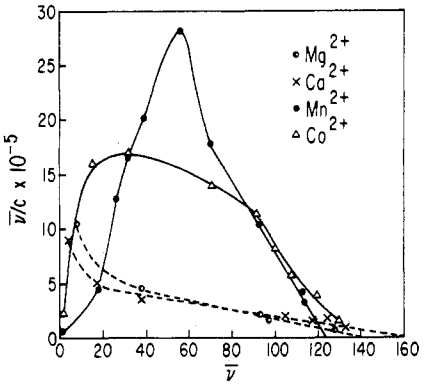

FIGURE 1: Scatchard plots for the binding of Mg^{2+} (●), Ca^{2+} (×), Mn^{2+} (●), and Co^{2+} (△) at pH 6.8 and 25°. Here $\bar{\nu}$ = ions bound per mole of phosvitin, and C = molar concentration of free ions in the solution.

Mg^{2+}, Mn^{2+}, or Co^{2+} prepared by dialysis equilibration were recorded in the wavelength range of 240–330 nm and were compared with phosvitin dialyzed against the sodium cacodylate buffer, pH 6.8 and 0.02 $\Gamma/2$. The temperature of the solutions was then raised to 37 and 60°, respectively, with the aid of a Haake circulating water bath (Type FJ) and the spectra were recorded again.

Magnesium and calcium were determined as described previously (Grizzuti and Perlmann, 1973).

Manganese was determined colorimetrically with formaldoxime following the general method described by Snell and Snell (1949). Cobalt was determined colorimetrically with 4-hydroxy-3-nitroso-1-naphthalenesulfonic acid according to a procedure described by Snell et al. (1959).

Phosvitin concentrations were based on nitrogen analysis performed by the Pregl microKjeldahl method using a mercuric catalyst (Hiller et al., 1948).

The pH of the solutions was measured either with the Radiometer pH meter, Model 4, or a Radiometer Titrator TT1a equipped with the Radiometer Scale Expander Type PHA 630 Ta, calibrated with the standard buffers recommended by Bates (1954). The differing amounts of divalent cations added to phosvitin produced only small variations in the pH values of the reaction mixtures. Furthermore, the change of pH in the temperature range of 25–60° did not exceed 0.06 pH unit.

Results

Binding Studies. Typical results of the binding of Mn^{2+} and Co^{2+} to phosvitin in the concentration range of 0.00002–0.0015 M at pH 6.8 and 25° are presented in Figure 1. Plotting our data according to Scatchard (1949), complex curves are obtained and not the simple straight lines to be expected if the binding occurred on identical independent sites along the phosvitin molecule, as we have found previously for Ca^{2+} and Mg^{2+}. For comparison these latter results already reported are included in Figure 1 (Grizzuti and Perlmann, 1973). It can be seen in this figure that the plots of $\bar{\nu}/C$ vs. $\bar{\nu}$ at low concentrations of bound Mn^{2+} (less than $\bar{\nu}$ = 50) or of Co^{2+} (less than 20) have a positive slope with a downward curvature indicating that, in the absence of aggregation of phosvitin, a possibility which we have not yet investigated, there is interaction between binding sites and the binding is cooperative involving a structural change in the macromolecule [Danchin and Guéron, 1970; Danchin, 1972; Giancotti et al., 1973].

At higher concentrations of bound Mn^{2+} and Co^{2+} no cooperativity exists. As shown in Figure 1, if more than 90 Mn^{2+} or Co^{2+} are bound by phosvitin the plots of $\bar{\nu}/C$ vs. $\bar{\nu}$ become linear and the total number of binding sites, n, and the binding constant, nk, may be estimated. Thus n = 128 and 138 for Mn^{2+} and Co^{2+}, respectively.

Table I: Number of Divalent Cation Binding Sites and Binding Constant, K, per Site.

Cations	No. of Sites: Weak (Independent)	No. of Sites: Strong (Cooperative)	$K \times 10^{-4}\ M^{-1}$: Weak	$K \times 10^{-4}\ M^{-1}$: Strong
Ca^{2+}	160	None	0.36	
Mg^{2+}	140	None	0.44	
Mn^{2+}	128	55	0.23	2.05
Co^{2+}	138	30	0.56	3.30

Both Mn^{2+} and Co^{2+} binding plots display transition regions. For Mn^{2+} it is between 50 and 90 ions bound per mole of phosvitin while for Co^{2+} it is shifted to a lower concentration of bound ions, i.e., between 20 and 90. The upward curvatures with negative slopes of the Scatchard plots are indicative of the influence of at least one other type of binding site with a different association constant.

In an attempt to establish the number of sites corresponding to the two types of binding, Mn^{2+} and Co^{2+} Scatchard plots were broken down according to the method of Danchin and Guéron (1970). Table I summarizes the results obtained and also includes, for comparison, those already reported for Ca^{2+} and Mg^{2+}.

Effect of Temperature on Binding. The dependence of the binding of Ca^{2+}, Mg^{2+}, Mn^{2+}, and Co^{2+} to phosvitin on temperature is illustrated in Figures 2 and 3. As the temperature is raised from 25 to 37 and 60°, respectively, the plots of $\bar{\nu}/C$ vs. $\bar{\nu}$ for Ca^{2+} and Mg^{2+} give essentially straight lines (Figure 2). For Ca^{2+}, n = 160, 170, and 160 at 25, 37, and 60°, whereas the changes observed for Mg^{2+} were n = 140, 140, and 160 at the above temperatures. Figure 3 illustrates the effect of temperature on the binding of Mn^{2+} and Co^{2+}. Although basically little variation is observed in the maximum number of ions bound, the cooperativity of the binding sites is enhanced considerably.

Effect of Binding of Divalent Cations to Phosvitin. VISCOSITY. In a previous communication (Grizzuti and Perlmann, 1970) we have shown that the presence of NaCl affects the viscosity of phosvitin. In view of these results the effect of the binding of divalent cations to phosvitin on the viscosity was investigated. In Figure 4 are presented the reduced specific viscosities, η_{sp}/C, of phosvitin in sodium cacodylate buffer (pH 6.8), $\Gamma/2$ = 0.02 and 0.1, and of phosvitin with 102 Mg^{2+}, 147 Ca^{2+}, 98 Mn^{2+}, and 112 Co^{2+} ions bound per mole, as a function of phosvitin concentration. In the presence of the cations, we could not measure viscosities at phosvitin concentrations higher than 0.6% due to the precipitation of 95% of this phosphoglycoprotein.

As shown in Figure 4, the reduced specific viscosity of phosvitin in the sodium cacodylate buffer of pH 6.8 at an ionic strength of 0.02 has typical polyelectrolyte characteristics caused by the strong electrostatic repulsion between the negatively charged phosphate groups of the protein. To repress this charge effect, the ionic strength of the buffer has to be increased to at least 0.1 to obtain a linear extrapolation of the reduced specific viscosity to a value of $[\eta]$ = 0.3 dl/g. It is, therefore, of particular interest to note that on binding of 100–140 divalent cations to phosvitin the spe-

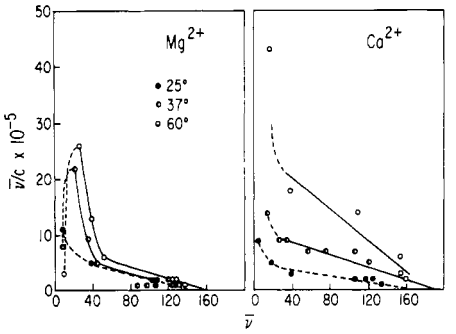

FIGURE 2: Temperature dependence of the Scatchard plots for the binding of Mg^{2+} and Ca^{2+} at pH 6.8: 25° (●), 37° (◐), and 60° (○).

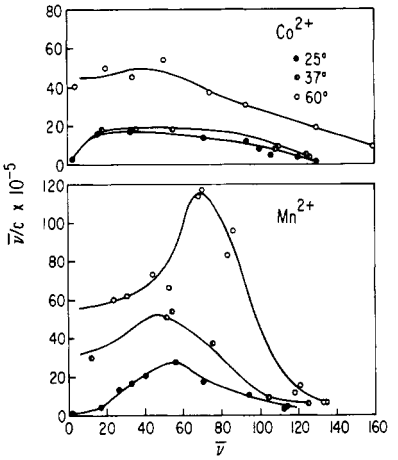

FIGURE 3: Temperature dependence of the Scatchard plots for the binding of Mn^{2+} and Co^{2+} at pH 6.8: 25° (●), 37° (◐), and 60° (○).

cific viscosities in the phosvitin concentration range of 0.02–0.6% decrease sharply. The binding of Mg^{2+} and Ca^{2+} ions decreases $[\eta]$ to 0.048 dl/g whereas the binding of Mn^{2+} and Co^{2+} causes a further depression of $[\eta]$ to 0.03 dl/g.

This decrease in the viscosity of phosvitin upon binding of Mg^{2+}, Ca^{2+}, Mn^{2+}, and Co^{2+} could be explained in terms of neutralization of the negative charges of the acidic groups of the phosvitin molecule by the divalent cations, thus reducing the electrostatic repulsion between them and allowing the molecule to compact. However, in view of our previously reported data (Grizzuti and Perlmann, 1970) to the effect that even ionic strengths as high as 0.5 only bring the $[\eta]$ down to 0.25 dl/g, neutralization of charges only can hardly account for the results seen in Figure 4 and some type of ion-specific effect should also be taken into consideration.

ABSORPTION SPECTRA. The ultraviolet absorption spectrum of phosvitin at 25° and at pH 6.8, in cacodylate buffer, $\Gamma/2 = 0.02$, is shown in Figure 5. It can be seen here that in the wavelength range of 330–240 nm there is a small maximum at 275 nm and also an indication of a much higher absorption peak below 240 nm. For a phosvitin solution containing 0.72 mg/ml, the maximum absorption at 275 nm is 0.367, which is quite small due to the low content of aromatic amino acids in phosvitin (Allerton and Perlmann, 1965).

In the same figure we plotted the spectra obtained after phosvitin had bound 120 Mg^{2+}, 100 Ca^{2+}, 116 Mn^{2+}, and 123 Co^{2+}. The main changes in the spectrum of phosvitin in the presence of these cations is a shift of the maximum at

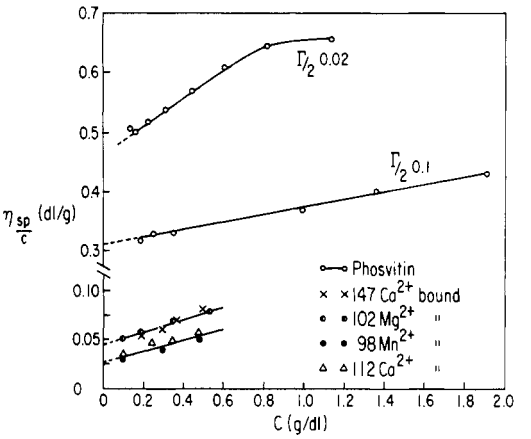

FIGURE 4: Dependence of the reduced specific viscosity, η_{sp}/C, of phosvitin on protein concentration at pH 6.8 and at $\Gamma/2 = 0.02$ and 0.1 (○), and of phosvitin with 147 Ca^{2+} (×), 102 Mg^{2+} (◐), 98 Mn^{2+} (●), and 112 Co^{2+} (△) bound at pH 6.8 and $\Gamma/2 = 0.02$ at 25°.

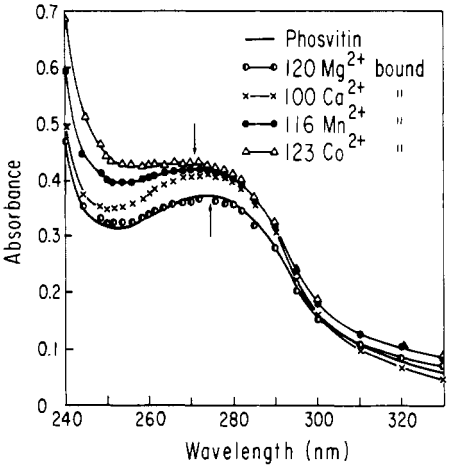

FIGURE 5: Ultraviolet absorption spectra of phosvitin (—) and of phosvitin with 120 Mg^{2+} (◐), 100 Ca^{2+} (×), 116 Mn^{2+} (●), and 123 Co^{2+} (△) bound at pH 6.8 and $\Gamma/2 = 0.02$ and 25°.

275 to 272 nm when the ion bound is Ca^{2+}, and to 270 nm when Mn^{2+} or Co^{2+} are bound. Simultaneously there is a flattening of the spectral region between 240 and 290 nm, which is specially marked with Co^{2+}, in which case the absorption at 270 nm is 0.432. Mg^{2+} is the only cation that does not have any effect on the phosvitin spectrum at 25°.

Upon increasing the temperature from 25 to 37 to 60°, the spectrum of phosvitin remains basically unchanged; in the presence of the various cations a slight increase in the absorbance occurs without a further blue shift of the absorption maximum. These spectral changes are reversible. On cooling of the solutions to 25°, the spectra recorded are identical with those obtained prior to heating of the solutions.

Optical Rotatory Dispersion and Circular Dichroism. As already reported (Grizzuti and Perlmann, 1973), the binding of divalent cations Mg^{2+} and Ca^{2+} has an influence on the ORD patterns and CD spectra of phosvitin.

In Figure 6A and B we have plotted the ORD and CD spectra corresponding to the binding of 112 Mn^{2+} and 130 Co^{2+} ions per mole of phosvitin, and we have also included, for purposes of comparison, those corresponding to the binding of Mg^{2+} and Ca^{2+} already reported. It can be seen that the effect of Mn^{2+} and Co^{2+} binding parallels very much that of Ca^{2+}, that is the reduced mean residue rota-

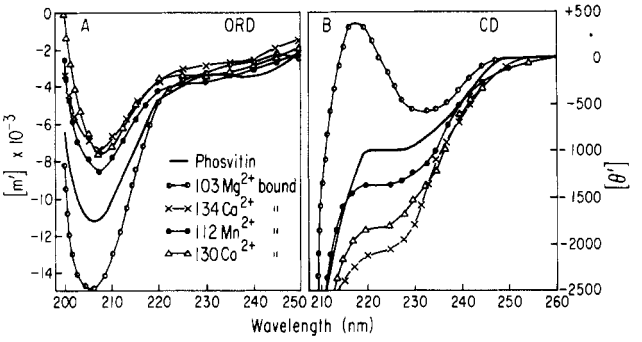

FIGURE 6: Effect of the binding of 103 Mg^{2+} (◐), 134 Ca^{2+} (×), 112 Mn^{2+} (●), and 130 Co^{2+} (△) on the optical rotatory dispersion and circular dichroism of phosvitin at pH 6.8 and $\Gamma/2 = 0.02$ and 25°.

tion, $[m']_{207}$, becomes less levorotatory, increasing to −8500 and −7500, respectively. A similar pattern is seen in the CD spectra where the reduced mean residue ellipticity, $[\theta']_{220}$, decreases to −1400 and −1750 for Mn^{2+} and Co^{2+}, respectively.

To further assess the different nature of the effect of the various cations on the conformation of phosvitin and also to estimate the minimum number of ions required to start the conformational change, we performed titrations of this phosphoglycoprotein in sodium cacodylate buffer, pH 6.8 and $\Gamma/2 = 0.02$, with 0.05 *M* $MgCl_2$ and $MnCl_2$. We followed $[m']_{207}$ and $[\theta']_{220}$ as a function of the moles of cation added per mole of phosvitin. An example of the type of curves obtained is shown in Figure 7. It is quite obvious from this figure that the conformational changes induced by Mg^{2+} and Mn^{2+} follow different paths, in addition to being in the opposite direction.

The phosvitin molecule is conformationally stable in the presence of up to 50 Mn^{2+} ions per mole of phosvitin; an increase in its concentration initiates a conformational change that is completed by the time 115 Mn^{2+} have been added. Further increases in Mn^{2+} concentration do not affect $[m']_{207} = -8000$. After 275 Mn^{2+} per phosvitin molecule have been added the protein starts to precipitate.

When Mg^{2+} is the titrant, no initial lag in the start of the conformational change is seen and the first transition is completed with 115 Mg^{2+} added per mole of phosvitin. A second transition occurs between 275 and 300 Mg^{2+} added with $[m']_{207}$ decreasing to −13,800. Concentrations as high as 750 Mg^{2+} per mole of phosvitin do not cause any further changes in $[m']_{207}$; above this, however, the protein precipitates. These same patterns are obtained with CD titrations.

At pH 6.8 the two hydroxyls of each of the 136 phosphate groups on the phosvitin molecule are ionized. Therefore it seems reasonable to state that the first conformational change is complete when one of the negative charges on each phosphate group is neutralized. No further change in conformation occurs until the second negative charge is also neutralized, at which time Mn^{2+} causes precipitation while Mg^{2+} causes a second transition to a conformation which is very stable even in the presence of 700 Mg^{2+} per phosvitin molecule.

Discussion

Thus far the biological function of phosphoproteins is unknown. However, a few suggestions have been made; for instance, enzymic phosphoryl transfer or energy storage may involve the participation of the phosphorylated amino acid residues of these proteins. Another suggestion is their participation in ion transport.

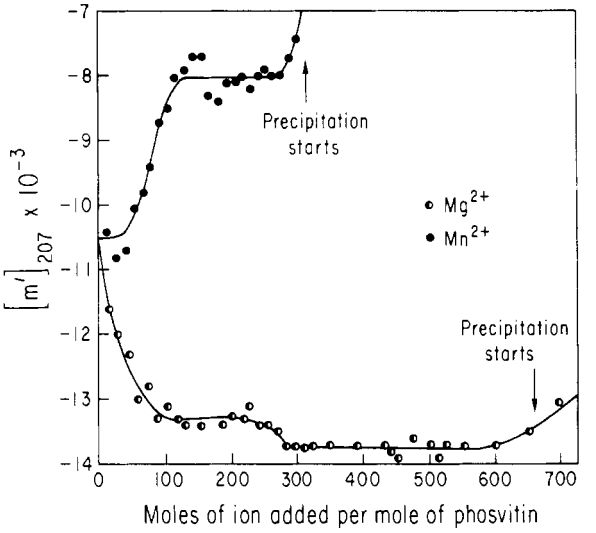

FIGURE 7: Dependence of $[m']_{207}$ of phosvitin on the stepwise addition of Mg^{2+} (◐) and Mn^{2+} (●) in sodium cacodylate buffer pH 6.8 and $\Gamma/2 = 0.02$ and at 25°.

Reports of interactions of phosvitin with several types of compounds like alkali metal ions, polylysines and protamines, cytochrome *c*, Acridine Orange, Fe^{2+}, Fe^{3+}, Mg^{2+}, and Ca^{2+} point to one or the other of the above suggestions [for specific references and a comprehensive survey, see Taborsky (1974)].

From our previous report (Grizzuti and Perlmann, 1973) and the present results it becomes evident that two types of antagonisms are observed in the interactions of Mg^{2+}, Ca^{2+}, Mn^{2+}, and Co^{2+} with phosvitin at pH 6.8. On one hand we have Mg^{2+} whose effects stand out against those of Ca^{2+}, Mn^{2+}, and Co^{2+}, and on the other we have Mg^{2+} and Ca^{2+} opposed to Mn^{2+} and Co^{2+}. In the first category we find the effects of Mg^{2+} on the ORD and CD spectra of phosvitin, the stability of the ultraviolet absorption spectra of phosvitin in the presence of Mg^{2+}, and the relative insensitivity of the Mg^{2+} binding to temperatures as high as 60°. In the second group we find the binding of Mg^{2+} and Ca^{2+} as opposed to the binding of Mn^{2+} and Co^{2+}, and also the lowering of the intrinsic viscosity of phosvitin; in this instance Mn^{2+} and Co^{2+} are more effective than Mg^{2+} and Ca^{2+}.

At this point it is not possible to explain how and why these differences arise. Considerations of ionic radii, ionic hydration, and protein hydration cannot be discarded. It is quite clear, however, that the conformation of phosvitin is strongly influenced by divalent cations at very low concentrations; to induce changes equivalent to those produced by Mg^{2+}, 50 times higher concentrations of Na^+ ions would be needed. Therefore it is not impossible that the divalent cations, Mg^{2+} in particular, may play an important role in the biological function of phosvitin.

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Interactions of Bilirubin and Other Ligands with Ligandin†

Kazuaki Kamisaka,‡ Irving Listowsky,*,§ Zenaida Gatmaitan, and Irwin M. Arias

ABSTRACT: Circular dichroism methods were used to study the structure of rat ligandin and the binding of organic anions to the protein. Ligandin has a highly ordered secondary structure with about 40% α helix, 15% β structure, and 45% random coil. Bilirubin binding occurred primarily at a single high affinity site on the protein. The binding constant for bilirubin ($5 \times 10^7\ M^{-1}$) was the highest among the ligands studied. The bilirubin–ligandin complex exhibited a well-defined circular dichroic spectrum with two major overlapping ellipticity bands of opposite sign in the bilirubin absorption region. This spectrum was virtually a mirror image of that of human or rat serum albumin–bilirubin complexes. Studies on the direct transfer of bilirubin from ligandin to rat serum albumin showed that association constants of bilirubin–ligandin complexes were approximately tenfold less than those of the bilirubin–albumin system. Ligandin exhibited a broad specificity with respect to the type of ligand bound. A series of organic anions including dyes used clinically for liver function tests, fatty acids, hormones, heme derivatives, bile acids, and other ligands that were considered likely to interact with ligandin, were examined. Most induced ellipticity changes consistent with competitive displacement of bilirubin from ligandin and relative affinities of these compounds for ligandin were determined based on their effectiveness in displacing the bilirubin. Some substances such as glutathione, conjugated sulfobromophthaleins and lithocholic acid bound to ligandin but induced anomalous spectral shifts, when added to ligandin–bilirubin complexes. Other compounds, including some that act as substrates for the glutathione transferase activity exhibited by ligandin, revealed no apparent competitive effects with respect to the bilirubin binding site.

Ligandin is an abundant cytoplasmic protein localized mainly in liver cells, proximal tubules of kidney, and nongoblet mucosal cells of the small intestine, in rats, monkey, and man (Levi et al., 1969a,b, Fleischner et al., 1972). Ligandin is a basic protein (pI = 9.1) with a molecular weight of 46,000 and consists of two apparently identical 23,000-dalton subunits (Litwack et al., 1971). It is considered to be a major determinant of the net flux of various organic anions from plasma into the liver (Arias, 1972). These include bilirubin, various dyes, (i.e., sulfobromophthalein, Indocyanine Green, Evans Blue), and metabolites. Ligandin also has glutathione transferase activity for selective substrates and is identical with glutathione transferase B (Habig et al., 1974). Several carcinogen metabolites bind covalently to ligandin (Litwack et al., 1971), whereas interactions with most other ligands are noncovalent.

An objective of the present report was to determine relative affinities of various ligands for ligandin. Competitive binding of these substances relative to bilirubin was analyzed by circular dichroism (CD) methods. Results with sulfobromophthalein using CD were correlated with estimates of affinity constants determined by equilibrium dialysis.

Experimental Section

Materials

Bilirubin was obtained from Eastman Corp., Rochester, N.Y., or Sigma Corp., St. Louis, Mo.; sulfobromophthalein and Indocyanine Green were purchased from Hyson, Westcott-Dunning, Inc., Baltimore, Md.; sodium penicillin and iodipamide were from E. R. Squibb Sons, Inc., New York, N.Y.; chloromycetin sodium succinate was from Parke-Davis Co., Detroit, Mich.; cortisol and oleic acid were from Applied Science Lab., Inc., State College, Pa.; hematoporphyrin, hemin, protoporphyrin, benzpyrene, *p*-aminohippuric acid, probenecid, cholic acid, chenodesoxycholic acid, deoxycholic acid, glycocholic acid, and lithocholic acid were from Sigma Corp., St. Louis, Mo.; glutathione (GSH re-

† From the Liver Research Center, Departments of Medicine (Division of Gastroenterology—Liver Diseases) and Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461. *Received November 13, 1974.* This work was supported by Grants AM 2019, 4465, 4479, and HL-11511 and Liver Center Grant 17702.

‡ Present address: 2nd Department of Internal Medicine, University of Tokyo.
§ Career Development Awardee (GM 19958) from the National Institute of General Medical Sciences, National Institutes of Health.