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Binding of Magnesium and Calcium Ions to the Phosphoglycoprotein Phosvitin†

Kärt Grizzuti and Gertrude E. Perlmann*

ABSTRACT: Dialysis equilibrium measurements were carried out at 25° at pH 6.5 and 4.5 to determine the binding of Mg^{2+} and Ca^{2+} by phosvitin. We find that at pH 6.5, in the concentration range of 0.2×10^{-3} to 2.0×10^{-3} M $MgCl_2$ or $CaCl_2$, phosvitin at 25° binds an average of 103 Mg^{2+} and 127 Ca^{2+}, respectively, whereas at pH 4.5 40 Mg^{2+} and 32 Ca^{2+} are bound. Optical rotatory dispersion and circular dichroism studies indicate that a conformational change occurs on interaction with the divalent cations. At pH 6.5, in the presence of Mg^{2+}, the mean residue rotation [m'] becomes more levorotatory, whereas interaction with Ca^{2+} has the opposite effect on [m'], implicating the importance of the ionic environment in controlling the polypeptide backbone conformation of phosvitin.

Studies of viscosity on the phosphoglycoprotein phosvitin have shown that this protein in some of its properties resembles a polyelectrolyte. Furthermore, it was also demonstrated that the presence of monovalent cations, Na^+ and K^+, has an effect on the optical rotatory dispersion (ORD) and circular dichroism (CD) (Grizzuti and Perlmann, 1970).

The purpose of the investigation described in this paper was to measure the binding of the divalent cations, Mg^{2+} and Ca^{2+}, by phosvitin and to study the effect of ion binding on the conformational characteristics of this protein. Dialysis equilibrium techniques and gel filtration have been used. We shall show that at the two pH values investigated the number of Ca^{2+} bound differs from that of Mg^{2+}, indicating a selectivity in the binding of divalent ions. In addition, a significant difference is found in the ORD patterns and CD spectra of this protein.

Materials and Methods

Materials. Phosvitin with a nitrogen content of 13.1% and phosphorus of 11.9% was isolated from fresh hens' eggs according to the procedure of Joubert and Cook (1958).

^{45}Ca was purchased as aqueous calcium chloride from Amersham/Searle; Aquasol, the scintillation counting fluid, was supplied by New England Nuclear Corp. Eriochrome Black T was purchased from Matheson, Coleman & Bell and Erio SE from Eastman Organic Chemicals. Sephadex G-25 was a product of Pharmacia. All other chemicals were reagent grade and were not further purified.

Methods. The binding of Ca^{2+} and Mg^{2+} was determined either by equilibrium dialysis at 25° or in some experiments by gel filtration on Sephadex G-25.

The *dialysis* experiments were carried out as follows: in the tests in which the protein concentrations were varied aliquots of a freshly prepared phosvitin solution (10 mg/ml) in either distilled water or sodium cacodylate buffer (pH 6.8), $\Gamma/2$ 0.02, were diluted to the desired protein concentration; 0.5 ml of each solution contained in bags of Visking sausage casing[1] were placed in large test tubes with 40 ml of the appropriate solvent. The tubes were placed on magnetic stirrers and mixing was obtained by gentle stirring. At specified time intervals the bags were withdrawn, carefully rinsed with distilled water, and wiped dry, and the contents were analyzed for protein and bound ion. Equilibrium at 25° was usually attained in 2–4 hr (see Results). Equilibrium controls consisting of Mg^{2+} or Ca^{2+}

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[1] Prior to use the dialyzing membranes were boiled in distilled water, followed by thorough rinsing and storage at 4°.

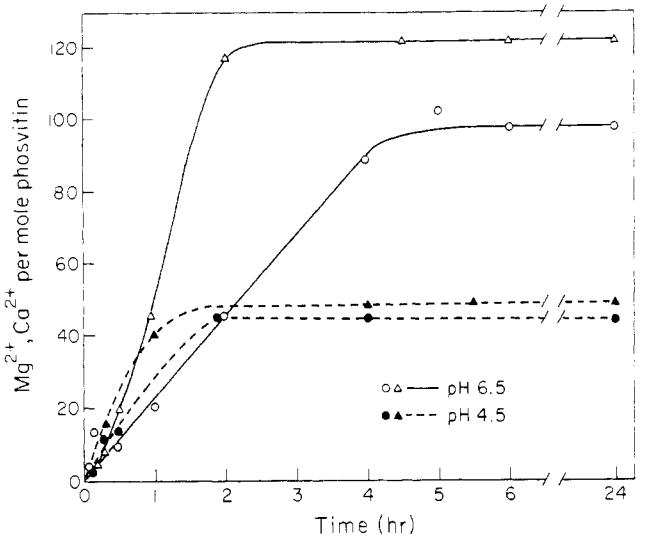

FIGURE 1: Time dependence of the binding of Mg^{2+} (●, ○) and Ca^{2+} (▲, △) at pH 4.5 and 6.5. Conditions of equilibrium dialysis: 0.54×10^{-4} M phosvitin and 2.1×10^{-3} M Mg^{2+} or Ca^{2+}.

solutions outside and buffer or distilled water inside the bag were frequently included in the experiments. Furthermore, the possibility that loss of phosvitin might occur by adsorption to the membrane was investigated. It was found that no significant adsorption took place in the range of the protein concentrations used.

An alternate procedure in which the protein concentration was kept constant but the ion concentration varied consisted in placing 9 ml of phosvitin solution (*e.g.*, 2.2 mg/ml) in a dialysis bag tied on one end but which had a tubing inserted at the other end to allow withdrawal of aliquots at specified time intervals. Dialysis was carried out against 90 ml of the solvent. This arrangement permitted simultaneous analyses of the amount of protein-bound ions and the optical measurements on the solutions.

Binding studies by *gel filtration* were carried out at 25° on a Sephadex G-25 column (1 × 60 cm) according to Hummel and Dreyer (1962). The column was equilibrated with the Mg^{2+} or Ca^{2+} solution of the desired concentration. Phosvitin was then dissolved in the same solvent which was also used as eluent. Chromatography was performed at constant flow rate and each fraction (1.5 or 2.8 ml) was analyzed for protein and Ca^{2+} or Mg^{2+}.

ORD and CD measurements were carried out at 25° in a Cary 60 recording spectropolarimeter equipped with the 6001 circular dichroism attachment as described previously (Grizzuti and Perlmann, 1970).

Analytical Methods

Calcium binding was determined as follows: (a) in the gel filtration experiments the binding of ^{45}Ca was followed by measuring the radioactivity of each fraction in a Packard Tri-Carb Model 3320 liquid scintillation counter or by flame photometry on an attachment FA-2 fitted to the Zeiss PMQII spectrophotometer;[2] (b) in the equilibrium dialysis experiments Ca^{2+} was determined colorimetrically using the dye Erio SE according to the procedure of Brush (1961).

Magnesium binding was determined colorimetrically with Eriochrome Black T (Smith, 1955), except that the 8% ammonium hydroxide and trichloroacetic acid were omitted from the reaction mixture. The dye solutions were always freshly prepared.

Phosvitin concentrations were based on phosphorus determinations as described by Allerton and Perlmann (1965) or on nitrogen analysis performed by the Pregl microKjeldahl method using a mercuric catalyst (Hiller *et al.*, 1948).

The *pH* of all solutions was measured with the Radiometer pH Meter Model 4, calibrated with the standard buffers recommended by Bates (1954). The differing amounts of Ca^{2+} and Mg^{2+} added to the phosvitin solutions produced small variations in the pH values of the reaction mixtures. The changes observed in the ORD and CD measurements, however, cannot be due to these pH changes (*cf.* Grizzuti and Perlmann, 1970). Therefore, the pH measured on addition of salt and reported in this paper represent average values.

TABLE 1: Binding of Mg^{2+} and Ca^{2+} to Phosvitin (0.54×10^{-4} M) at pH 4.5.

Cation Concn, M × 10^3			Cation Bound/Mol of Phosvitin ($\bar{\nu}$)	$\bar{\nu}/C \times 10^{-4}$
Initial Outside	Final Inside	Bound		
		Mg^{2+}		
0.2	2.3	2.1	39	19.5
0.8	3.2	2.4	44	5.4
2.4	4.9	2.5	46	1.9
3.9	5.8	1.9	35	0.9
		Ca^{2+}		
0.1	2.1	2.0	37	37.0
1.3	3.0	1.7	31	2.4
1.9	3.5	1.6	30	1.6
2.1	3.6	1.5	28	1.4

Results

Our first measurements were directed toward establishing the rate and extent of binding of Ca^{2+} and Mg^{2+} to phosvitin. As shown in Figure 1, in the experimental setup chosen (0.54×10^{-4} M protein and 2.1×10^{-3} M Ca^{2+} or Mg^{2+}) equilibrium is attained in 2–4 hr. The noteworthy feature of Figure 1, however, is that equilibrium with Ca^{2+} at the two pH values studied is reached faster than with Mg^{2+}. Thus for Ca^{2+} $k_{pH\ 6.5} = 1.03$ and $k_{pH\ 4.5} = 0.7$, whereas for Mg^{2+} $k_{pH\ 6.5} = 0.39$ and $k_{pH\ 4.5} = 0.37$.[3] Furthermore, the maximum amount of Ca^{2+} bound at pH 6.5 exceeds that of Mg^{2+}.

A more detailed analysis of our investigation is presented in Tables I and II. In the first column is listed the initial concentration, C, of the cations; the second and third columns give the final cation concentration of the solution in the dialysis bag and the moles of cation bound. The last two columns list the number of cations bound per molecule of phosvitin, $\bar{\nu}$, and the corresponding values of $\bar{\nu}/C$. The results show that at pH 4.5 (water) at the constant phosvitin concentration (0.54×10^{-4} M) but varying salt concentrations, the amount of Mg^{2+} bound varies very little and an average value of 40 Mg^{2+}/mol of phosvitin was established whereas under similar experimental conditions only 32 Ca^{2+} are bound. One has to keep in

[2] Our thanks go to Dr. Asher Haymovits for his advice and the use of his flame photometer.

[3] $k = d\bar{\nu}/dt$, $\bar{\nu}$ *cf.* Tables I and II, the subscript refers to the pH of each experiment.

TABLE II: Binding of Mg^{2+} and Ca^{2+} to Phosvitin (0.54×10^{-4} M) at pH 6.5.

Cation Concn, M × 10^3			Cation Bound/Mol of Phosvitin ($\bar{\nu}$)	$\bar{\nu}/C \times 10^{-4}$
Initial Outside	Final Inside	Bound		
		Mg^{2+}		
0.02	0.37	0.38	7	104
0.1	1.0	0.92	17	66
0.2	2.3	2.0	37	46
0.8	5.6	5.0	92	21
1.5	5.6	4.1	76	29
2.3	7.2	6.8	127	16
		Ca^{2+}		
0.02	0.32	0.30	6	90
0.1	0.97	0.92	17	51
0.2	2.3	1.9	36	44
0.7	7.2	6.5	120	16
1.5	7.2	5.7	106	20
2.0	8.7	6.9	128	8

mind that at this pH only one hydroxyl of each phosphate group is ionized. In contrast, however, at pH 6.5, where most of the hydroxyls with the pK' of 6.8 have lost their protons, the binding is appreciably higher (Table II), *i.e.*, *ca.* 103 Mg^{2+} and *ca.* 127 Ca^{2+}. These average values agree well with those of experiments in which the protein concentration was varied about tenfold but the cation concentration kept constant

Binding experiments at pH 4.5 were also performed by gel filtration on Sephadex G-25 (see Methods section). The number of Mg^{2+} and Ca^{2+} bound per phosvitin molecule obtained with this procedure corresponded to 38 Mg^{2+} and 30 Ca^{2+}, values in good agreement with those obtained with the equilibrium dialysis.

To assess the binding constants and the number of sites available, the values of $\bar{\nu}/C$ (column 5) were plotted against $\bar{\nu}$ (column 4). The plot of $\bar{\nu}/C$ *vs.* $\bar{\nu}$ (Scatchard, 1949) given in Figure 2 suggests that the binding sites detected over the concentration range of 0.0002–0.002 M at pH 6.5 are of similar affinity. Since binding data at higher concentrations of Ca^{2+} and Mg^{2+} cannot be obtained due to precipitation of phosvitin by these cations, linear extrapolation of this plot to the abscissa reveals that n = 140 and 160 for Mg^{2+} and Ca^{2+}, respectively. It is of interest to note that phosvitin has 136 phosphoamino acids (phosphoserine and phosphothreonine) and 31 dicarboxylic amino acid residues[4] which at pH 6.8 should be fully ionized (Allerton and Perlmann, 1965). Thus n = 160 for Ca^{2+} is in fair agreement with the analytical data derived from amino acid analyses.

Linear extrapolation of the Scatchard plot to the ordinate gives values of nK = *ca.* 61.5×10^4 for Mg^{2+} and nK = *ca.* 56.0×10^4 for Ca^{2+} (K = association constant per binding site). These estimates are offered tentatively because the presence of a few binding sites of very high affinity may be the cause of the upward trend of the plot near the ordinate (*cf.* Figure 2); however, one should keep in mind that this region corresponds to a concentration range of cations too low to give reliable binding data.

Optical Rotatory Dispersion and Circular Dichroism. In a

[4] No amide determinations have been performed on phosvitin.

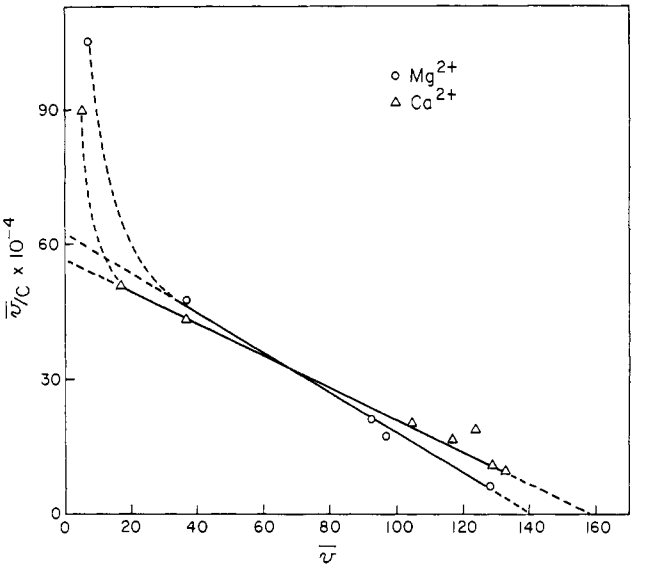

FIGURE 2: Scatchard plots for the binding of Mg^{2+} (O) and Ca^{2+} (Δ) at pH 6.5.

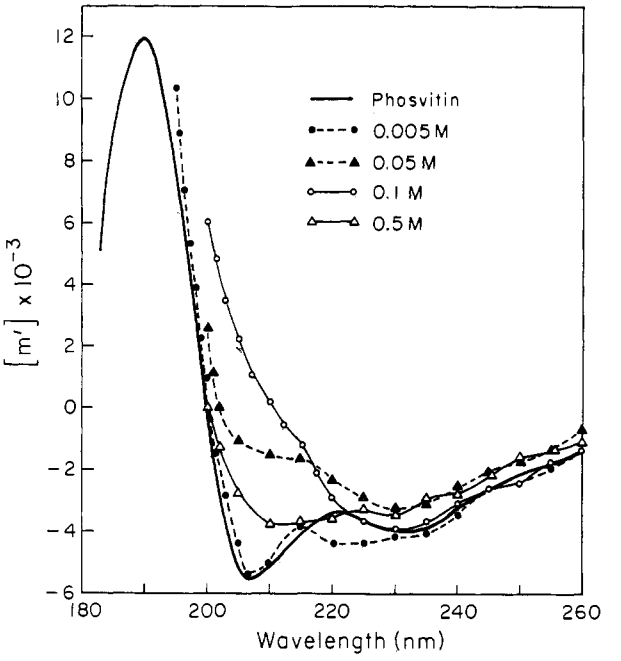

FIGURE 3: Dependence of the ORD of phosvitin at pH 4.0 on $MgCl_2$ concentration. Phosvitin dissolved and dialyzed against: water (——); $MgCl_2$, 0.005 M (●- - - - -●), 0.05 M (▲- - - -▲), 0.1 M (O—O), and 0.5 M (Δ—Δ).

previous communication (Grizzuti and Perlmann, 1970) we have shown that the presence of NaCl affects the ORD patterns and CD spectra of phosvitin. Similarly, $MgCl_2$ and $CaCl_2$ affect the optical properties of phosvitin. The changes observed are not only dependent on the nature of the cation added but also on the pretreatment of the solutions used for the measurements.

We found that when phosvitin is dissolved in $MgCl_2$ or $CaCl_2$ in a concentration range of 0.005–0.5 M (pH 4.5), the reduced mean residue rotation $[m']_{207}$[5] at the principal trough at 207 nm varies only slightly within the range of −5000 to

[5] Abbreviations used are [m'], reduced mean residue rotation; [θ'], reduced mean residue ellipticity.

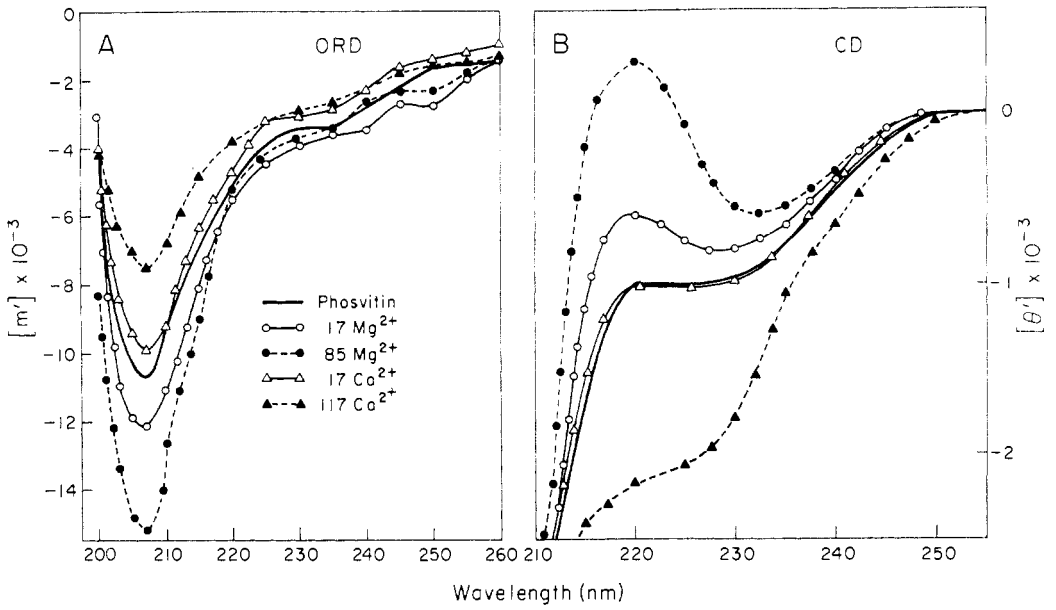

FIGURE 4: Effect of the binding of Mg^{2+} and Ca^{2+} on the ORD (A) and CD (B) of phosvitin at pH 6.5. Phosvitin dialyzed against: water (—); $MgCl_2$, 0.0001 M (○—○) *17* Mg^{2+} bound, 0.0023 M (●- - - -●) *85* Mg^{2+} bound; $CaCl_2$, 0.001 M (△— -△) *17* Ca^{2+} bound and 0.0020 M (▲- - - -▲) *117* Ca^{2+} bound.

−5700. In contrast, if phosvitin is dissolved in a $MgCl_2$ solution and dialyzed against the same solvent, $[m']_{207}$ becomes less levorotatory and the minimum disappears at $MgCl_2$ concentrations above 0.01 M (Figure 3). No significant changes of $[m']_{233}$ at the minor trough at 233 nm of the ORD patterns occur.

When phosvitin is dissolved in sodium cacodylate buffer of pH 6.8, Γ/2 0.02 containing $MgCl_2$ or $CaCl_2$, the ORD patterns are drastically affected. $[m']_{205}$ without added salt is −10,400. In the presence of 0.2 M $MgCl_2$, $[m']_{205}$ is positive, *i.e.*, 2000–3000, whereas on increasing the Mg^{2+} concentration $[m']_{205}$ becomes more levorotatory; $[m']_{205}$ = −15,600 at a Mg^{2+} concentration of 0.6 M.

In view of these results and in an attempt to obtain a direct comparison of the optical measurements and the binding experiments, phosvitin was dissolved in the pH 6.8 cacodylate buffer and dialyzed against the same buffer containing varying amounts of $MgCl_2$ and $CaCl_2$. This experimental setup enabled us to determine the Ca^{2+} and Mg^{2+} bound and to perform the ORD and CD measurements on the same solutions.

The dependence of the ORD and CD spectra on the type and number of cations bound is shown in Figure 4A,B. As the number of Mg^{2+} bound increases from 17 to 85 per mol of protein, $[m']_{207}$ decreases from −10,800 to −15,000. On binding of 117 Ca^{2+}/phosvitin molecule, $[m']$ becomes less levorotatory, *i.e.*, $[m']_{207}$ = −7500.

We have shown previously that in water or sodium phosphate buffer (pH 7.7), phosvitin has a strong negative dichroic band at 198 nm and a weaker band at 220 nm (Grizzuti and Perlmann, 1970). Owing to the high absorption of cacodylate in the far-ultraviolet region, the CD measurements could not be extended below 210 nm. Thus the spectra shown in Figure 4B reflect alterations in the weak dichroic band above 217 nm. However, a marked difference exists between Ca^{2+} and Mg^{2+}. On binding of 85 Mg^{2+}, $[\theta']_{217}$ = 300, whereas in the presence of 117 bound Ca^{2+} $[\theta']_{217}$ has a negative value.

Discussion

Before attempting to interpret the results presented in this article, it appears worthwhile to summarize the main observations made, and to indicate some of the chemical and conformational characteristics of phosvitin.

From the results of equilibrium dialysis experiments it was established that phosvitin in the pH range of 6.5 to 6.8 binds *ca.* 127 Ca^{2+} and 103 Mg^{2+}, respectively, whereas at pH 4.5 only 31 Ca^{2+} and 40 Mg^{2+} are bound.

This difference in binding of cations in the two pH regions is easily understood if one recalls that phosvitin, a protein with a molecular weight of 40,000, contains 136 phosphoamino acids (54% of its constituent). In neutral solution most of the phosphate groups are dianionic. In the pH range of 4.0–5.0, on the other hand, only one hydroxyl of each phosphate group is ionized, in addition to the 31 carboxyls of the dicarboxylic amino acid residues (12%). Although the binding of cations by phosvitin probably involves electrostatic interactions between the negatively charged side chains of the protein and the positive cations, one has to keep in mind that not all charged groups of phosvitin are equally available since sequences of three to eight phosphoserine residues linked covalently are present in this protein (Williams and Sanger, 1959; Shainkin and Perlmann, 1971). Thus, from the relative specific localizations of charged groups binding sites of different affinity to cations may arise and explain the selectivity observed.

The extent of binding of Ca^{2+} at pH 6.5, as derived from the Scatchard plot (Figure 2), is in agreement with the number of potential binding sites available. The difference between Ca^{2+} and Mg^{2+} bound, *i.e.*, 160 and 140, respectively, may possibly be understood if one assumes that 140 groups form loose combinations of the ordinary electrostatic type whereas phosvitin participates in "true" complex form with 20 Ca^{2+} but not with Mg^{2+}. It is, therefore, not unlikely that the increased specific combination of Ca^{2+} with phosvitin may be of biological significance. Unfortunately, this study could not be extended to a more alkaline pH region since at pH values above 8.0 β elimination occurs and dehydroalanine is formed at the expense of phosphoserine (Mecham and Olcott, 1949).

For the sake of completeness, data obtained at pH 4.5 are also included. We have not been able to handle these results on a formal basis, which is explained in part by the apparent incomplete dissociation of the phosphate groups.

If we now consider the ORD and CD spectra of phosvitin at pH 6.5, it appears that Ca^{2+} and Mg^{2+} affect the optical properties of the protein (Figure 4A,B). Although Mg^{2+} has a similar effect as previously observed for Na^{+} (Grizzuti and Perlmann, 1970), $[m']_{205}$ becomes less levorotatory when 17 or 117 Ca^{2+} is bound by phosvitin. Similarly, the fine structure of the dichroic bands in the wavelength range of 210–250 nm is affected in an adverse manner by Ca^{2+}. Thus these cations exert different effects on the spatial structure of the side chains of the phosvitin molecule.

In conclusion we should like to state that, although phosvitin in the pH range of 6.5–9.0 has an "unordered" conformation, ORD and CD measurements reveal that the nature of the counterions is of importance in controlling the polypeptide backbone conformation.

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Molecular Properties of the Cholinergic Receptor Purified from *Electrophorus electricus*†

Greg Biesecker

ABSTRACT: The cholinergic receptor protein from *Electrophorus electricus* was identified by the specific binding of a radioactively labeled snake neurotoxin, cobrotoxin, from *Naja naja atra*, solubilized from membrane fragments of the eel electric organ with detergent, and was purified on DEAE-cellulose and by affinity chromatography. The purified protein contained $2.2 \pm 0.3 \times 10^5$ g of protein/mol of toxin binding sites, gave a single band on an isoelectric focusing gel, and contained a single major polypeptide with a mol wt of 44,000. Results of cross-linking the tritiated toxin–receptor complex indicate that the mol wt 44,000 polypeptide is part of a mol wt 260,000 receptor molecule which binds the toxin.

According to the present theory of chemical transmission at cholinergic synapses (Eccles, 1964; Katz, 1969) depolarization of the postsynaptic membrane is initiated by the interaction of acetylcholine with a specific membrane component, the cholinergic receptor (AChR).[1] Numerous small molecules which either block or mimic the action of acetylcholine are thought to compete for a binding site on the receptor. In recent years small polypeptide neurotoxins which at very low concentrations irreversibly block synaptic transmission (Lee *et al.*, 1967; Lester, 1970) have been isolated from many snake venoms. Since their action is inhibited by cholinergic ligands, the snake neurotoxins are assumed to bind specifically to the receptor.

Because of its physiological importance, there have been many attempts to isolate and purify AChR (review articles: Rang, 1971; O'Brien *et al.*, 1972). Although the most direct method to identify the receptor is by its interaction with acetylcholine (Eldefrawi *et al.*, 1972), it is difficult to separate specific binding to the receptor from binding to acetylcholinesterase (whose catalytic sites must be blocked) and choline acetyltransferase and from nonspecific binding to other proteins. The much more specific binding of snake neurotoxins has proven a more reliable and easier means of identifying the cholinergic receptor. In addition to their irreversible actions *in vivo*, the snake neurotoxins specifically block the binding of acetylcholine (Eldefrawi *et al.*, 1972) and decamethonium (Changeux *et al.*, 1970) to the solubilized receptor and are in turn inhibited by several other cholinergic ligands (Meunier *et al.*, 1972).

Physical properties of crude preparations of detergent solubilized receptor labeled with radioactive neurotoxins have been investigated (Miledi *et al.*, 1971; Raftery *et al.*, 1971; Meunier *et al.*, 1971), and partial purification of the receptor has been achieved using isoelectric focusing (Eldefrawi and Eldefrawi, 1972) and several different affinity columns (Olsen *et al.*, 1972; Karlsson *et al.*, 1972; Schmidt and Raftery, 1973).

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[1] Abbreviation used is: AChR, acetylcholine receptor.