An Electrophoretic Study of the Reversible Binding of Phosphate to Ovalbumin

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An electrophoretic method is described for the measurement of relatively weak interaction of ions with proteins, and illustrated with the ovalbumin–phosphate system in 0.1*M* buffers, pH 6.1. Results indicate that under these conditions the interaction of a single dibasic phosphate ion with ovalbumin is described by an association constant of approx. 250 M^{-1} .

In many physicochemical investigations at neutral pH uni-univalent buffer systems, such as cacodylate or Tris/HCl, are used to obviate possible interactions of bivalent ions with proteins. However, since phosphate contributes significantly to the buffering capacity of the biological environment in which proteins and enzymes function, interactions of proteins with bivalent phosphate ion should clearly be studied instead of avoided. The relatively high concentration of phosphate (20–30 mM) *in vivo* (Newbold & Scopes, 1967) ensures that even weak protein–phosphate interactions with association constants in the range $10\text{--}100\text{ M}^{-1}$ can assume biological significance. Measurement of such equilibrium constants poses considerable practical difficulties, but the need for suitable methodology is emphasized by, for example, the pronounced enzymic activation of lactate dehydrogenase by phosphate (Winer & Schwert, 1958; Lovell & Winzor, 1974), and also the effect of phosphate on the isoelectric points of glyceraldehyde 3-phosphate dehydrogenase (Velick & Hayes, 1953) and α -chymotrypsin (Rao & Kegeles, 1958).

Development of an electrophoretic method for quantitative evaluation of protein–phosphate interactions is the aim of the present investigation. Two factors dictated the selection of ovalbumin as the model protein system. First, its greater negative electrophoretic mobility in phosphate than in uni-univalent buffer with identical pH is well established (Longworth, 1941). Secondly, the measured mobility differences between the A_1 , A_2 and A_3 components,

Theory

Consider a system $A + pS \rightleftharpoons AS_p$, where A is a macromolecular acceptor with p sites for charged ligand S (phosphate in the present context). After exhaustive dialysis of an acceptor–ligand mixture against a ligand solution with known molar concentration, $[S]$, the mixture is subjected to moving-boundary electrophoresis. Subject to the validity of assuming that the median bisector of the observed reaction boundary (descending side) identifies the position of the boundary in A constituent (Nichol & Ogston, 1965), it is possible to determine the constituent mobility of acceptor $\bar{u}_A$, which is defined as:

$$\bar{u}_A = \frac{\sum_{i=0}^p u_{AS_i} [AS_i]}{\sum_{i=0}^p [AS_i]} \quad (1)$$

The molar concentrations of the various acceptor–ligand complexes are given by expressions for the appropriate equilibria (eqn. 2).

Combination of eqns. (1) and (2) yields:

$$\bar{u}_A = \frac{u_A [A] + u_{AS} K_1 [A][S] + u_{AS_2} K_1 K_2 [A][S]^2 + \dots + u_{AS_p} K_1 K_2 \dots K_p [A][S]^p}{[A] + K_1 [A][S] + K_1 K_2 [A][S]^2 + \dots + K_1 K_2 \dots K_p [A][S]^p} \quad (3)$$

which differ only with respect to the number of phosphate groups attached (Perlmann, 1952, 1953), provide some indication of the likely change in mobility to result from attachment of an additional phosphate group.

If the ligand-binding sites are considered to be equivalent and independent, the equilibrium constants are interrelated by the expression:

$$K_i = (p - i + 1) K_A / i \quad (4)$$

where K_A is the intrinsic binding constant (Klotz, 1946). With these substitutions eqn. (3) becomes:

$$\bar{u}_A = \frac{u_A + u_{AS}pK_A[S] + u_{AS_2}\frac{p(p-1)}{2!}K_A^2[S]^2 + \dots + u_{AS_p}K_A^p[S]^p}{1 + pK_A[S] + \frac{p(p-1)}{2!}K_A^2[S]^2 + \dots + K_A^p[S]^p} \quad (5)$$

We now introduce the assumption that ligand binding changes the charge by a fixed amount for each molecule of ligand bound, without any change in Stokes radius of acceptor (Smith & Briggs, 1950) i.e. that:

$$u_{AS_i} = u_A + i\delta = u_A(1 + i\delta) \quad (6)$$

where δ is the incremental change in mobility expressed as a fraction of the mobility of uncomplexed acceptor. Substitution of eqn. (6) into eqn. (5), together with rearrangement and simplification, yields:

$$\frac{\bar{u}_A - u_A}{u_A} = \frac{p\delta K_A[S](1 + K_A[S])^{p-1}}{(1 + K_A[S])^p} = \frac{p\delta K_A[S]}{1 + K_A[S]} \quad (7)$$

The product $p\delta$ is a constant, representing the maximum fractional increase in mobility of acceptor, since from eqn. (6) it may be shown that:

$$p\delta = (u_{AS_p} - u_A)/u_A \quad (8)$$

Thus eqn. (7) describes a rectangular hyperbola defined by the two parameters K_A and the product $p\delta$: several methods (Klotz, 1946; Scatchard, 1949; Eisenthal & Cornish-Bowden, 1974) are available for their evaluation. The mobility increment $u_A p\delta$ may be converted into a charge increment by recourse to the Henry (1931) or Abramson *et al.* (1939) treatments of electrophoretic mobility, whereupon knowledge of the charge on S and the charge difference between A and AS_p may allow a tentative assignment of the value of p .

A point of interest in relation to eqn. (7) is the lack of dependence of $\bar{u}_A$ on acceptor concentration. This seems to imply that zonal-electrophoresis runs, with their inevitable spreading and consequent dilution, should suffice for the determination of K_A . However, application of eqn. (7) requires use of [S], which is a function of [A] (or total acceptor concentration) at any given position in the migrating system; but, provided that $[S] \gg [A]$, [S] remains essentially constant during migration. Hence zonal runs (paper electrophoresis or gel electrophoresis) performed with low loadings of acceptor that has been dialysed against ligand also offer, in principle, a means of evaluating approximate values of K_A and $p\delta$. Indeed, zonal electrophoresis has been

used to study the binding of metal ions to bovine serum albumin (Waldmann-Meyer, 1960).

Experimental

Crystalline ovalbumin (grade V) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Solutions of ovalbumin (approx. 75mg in 10ml) were obtained by direct dissolution of protein in the appropriate buffer (I0.10, pH6.1), followed by dialysis for 16h at 4°C against 500ml of the same buffer. Buffer pH values were measured at 20°C on a Pye model 291 pH-meter, by using an expanded pH-scale for comparison with a standard phosphate buffer, pH6.88 (0.0249M-KH₂PO₄/0.0249M-Na₂HPO₄).

Electrophoresis experiments were conducted on the dialysed solutions in the 15mm cell assembly of a Perkin-Elmer model 238 electrophoresis apparatus. The temperature of the bath was maintained at 4°C by means of a thermostatically controlled external refrigeration system. However, measured mobilities refer to a temperature of 0°C, the temperature at which the resistivities of protein solutions were measured with a Leeds and Northrup precision AC resistance bridge and a Shedlovsky-type conductivity cell.

Results

The results of moving-boundary electrophoresis at pH6.1 of ovalbumin in buffers (I0.10) containing various concentrations of phosphate are summarized in Fig. 1, about which several points should be noted. (i) A 5:1 molar ratio of monobasic to dibasic sodium phosphate was used to prepare the various phosphate buffers, the required ionic strength (0.10) being obtained by adding NaCl. A mixture of sodium cacodylate and cacodylic acid (both 0.02M) was used as the buffer system for the determination of u_A , the mobility of ovalbumin in the absence of phosphate. (ii) Since Longworth (1941) has noted that substitution of one univalent buffer salt for another, or for the neutral salt NaCl, does not result in appreciable mobility change, the observed variation in mobility with increasing phosphate concentration is attributed to binding of the bivalent phosphate ion; the abscissa of Fig. 1 is therefore expressed in terms of the concentration of Na₂HPO₄, rather than in terms of total phosphate concentration. (iii) The electrophoretic mobilities used to calculate

Fig. 1. Dependence of the electrophoretic mobility of ovalbumin on phosphate concentration

Results of moving-boundary-electrophoresis experiments performed on ovalbumin solutions (7.5 mg/ml) dialysed against buffer (pH 6.1, I 0.10) containing the appropriate concentration of phosphate are plotted in the format suggested by eqn. (7): ●, present results; ■, data of Longworth (1941). The two curves represent different estimates of the rectangular hyperbola that describes the results (see the text).

the binding parameter $(\bar{u}_A - u_A)/u_A$ are the mean of results from two to four experiments in each case, and refer to migration of the centroid (Longworth, 1943) of the descending-boundary system; no significant changes in the shape of the patterns were observed. (iv) Our data are in good agreement with the less extensive findings of Longworth (1941), whose results are also included in Fig. 1. (v) The preferential solvation of ovalbumin by phosphate ions is adequately described by a rectangular hyperbola, the form of dependence predicted by eqn. (7) for a system with a reversible phosphate binding as the source of the preferential solvation. (vi) Results have been tested for conformity with eqn. (7) by the method of Eisenthal & Cornish-Bowden (1974), which avoids the use of transformed variables. The solid curve describes the theoretical relationship for the numerically most stable and statistically least biased estimate of $p\delta$ and K_A , namely 0.163 and 200 M^{-1} respectively. By use of the centre of 'maximum probability' regions (Cornish-Bowden & Eisenthal, 1974) a slightly higher value of K_A (280 M^{-1}) and a lower $p\delta$ (0.143) are obtained (broken curve of Fig. 1).

On the basis of these estimates of $p\delta$, the difference between the mobilities of ovalbumin and the ovalbumin-phosphate complex is either 0.72×10^{-5} or $0.63 \times 10^{-5} \text{ cm}^2 \cdot \text{s}^{-1} \cdot \text{V}^{-1}$, values that are fairly similar to the differences observed at this pH (Perlmann, 1952, 1953) between the A_1 , A_2 and A_3 components of ovalbumin (0.5×10^{-5} – $0.6 \times 10^{-5} \text{ cm}^2 \cdot \text{s}^{-1} \cdot \text{V}^{-1}$). Since Perlmann (1952, 1953) has shown that these three components differ only with respect

to the number of phosphate groups present (two, one and zero), we attribute the faster migration of ovalbumin in phosphate buffers at pH 6.1 (I 0.10) to reversible binding of an additional dibasic phosphate ion described by an equilibrium constant of approx. 250 M^{-1} .

Discussion

Moving-boundary electrophoresis has been in use as a method of investigating interactions between macroions and charged ligands for a quarter of a century (Smith & Briggs, 1950; Alberty & Marvin, 1950). However, the present theoretical treatment represents a considerable simplification of the manner in which the data may be treated. Previously, experiments have been of different design inasmuch as charged ligand is usually added to the protein solution after dialysis, whereupon two boundaries (apart from the boundary anomaly) are observed in each limb of the electrophoresis cell. The free concentration of ligand is then obtained from the expression:

$$[S] = \frac{[\bar{S}](\bar{u}_S - \bar{u}_A) - [\bar{A}](\bar{u}_A - u_A)}{(u_S - u_A)} \quad (9)$$

which requires accurate knowledge of the total concentrations of the two reactants ($[\bar{A}]$, $[\bar{S}]$), and of the constituent and free mobilities of ligand ($\bar{u}_S$, u_S) in addition to $\bar{u}_A$ and u_A . In situations where there is no shortage of charged ligand, establishment of the free ligand concentration in the mixture by dialysis provides the experimenter with a much simpler analysis. In this connexion we note that Cann (1970) has recommended that $\bar{u}_A$ be obtained by the present method, but has retained eqn. (9) for evaluation of $[S]$ and hence of the Klotz (1946) binding parameter $\{([\bar{S}] - [S])/[\bar{A}]\}$. Clearly, analysis of $(\bar{u}_A, [S])$ data by means of eqn. (7) is much simpler and should be potentially more accurate by virtue of the fewer parameters that require evaluation.

A source of criticism of this work, and also of all previous electrophoretic investigations of interactions (e.g. Smith & Briggs, 1950; Longworth, 1959; Cann & Klapper, 1961; Nichol *et al.*, 1973), is the implicit assumption that the median bisector of an observed reaction boundary coincides with that of the boundary in the constituent that appears or disappears across the boundary (Nichol & Ogston, 1965); this assumption is encountered whenever refractometric optical systems are used. In the present context the contribution of ovalbumin to the Kohlrausch regulating function (Longworth, 1959) is approx. 4%, and hence the change in phosphate concentration across the descending reaction boundary gives rise to a negative refractive-index gradient that is 5–10% of the positive gradient

caused by the change due to protein concentration; its neglect should not be a serious source of error for the present reaction boundaries, which were essentially unimodal. The only unequivocal solution to this problem is analysis of the migration patterns by a procedure specific for the macromolecular constituent. In principle, absorption optics would provide such an analysis provided that the spectra of the protein and of all complexes AS_p exhibit a common isosbestic point, but this latter requirement may well prevent its universal application.

The previous reluctance to use $\bar{u}_A$ directly almost certainly stems from the realization that the difference between the mobilities of A and AS_p does not necessarily correspond to that expected on the basis of p additional charged ligand groups because of concomitant changes in binding of other buffer constituents (Longworth, 1959); in the present context the binding of a bivalent phosphate ion may well cause changes in the binding of univalent ions and also affect the ionization of amino acid residues. However, the assumption made in application of eqn. (7), and indeed of eqn. (9), is that the mobility difference, irrespective of its magnitude, is constant for successive ligand additions. Because only a single phosphate attachment is implicated in the present system, the ovalbumin results are not subject to this assumption, which, however, seems to apply to the bovine serum albumin–Methyl Orange system; Fig. 2 of Smith & Briggs (1950) shows a linear dependence of $\bar{u}_A$ on the Klotz (1946) binding parameter for this system with p approx. 20.

A valid criticism of the application of the present theory to the ovalbumin–phosphate system is the variation in the concentrations of the other buffer constituents that is made to accommodate the inclusion of phosphate. For example, an electrophoretic experiment with 0.015M- Na_2HPO_4 required elimination of all of the chloride and cacodylate that was present for the determination of u_A . Clearly the validity of assuming the same value of u_A for the two experiments can be questioned, but there seems to be no alternative in investigations of such weak interactions. To have retained the original buffer constituents at constant concentrations and to have made phosphate an additional component would have resulted in a progressive increase in I from 0.1 to 0.2, an unacceptable situation because of the dependence of mobility on ionic strength (Henry, 1931; Abramson *et al.*, 1939). By virtue of the practice adopted we have implied the absence of pronounced changes in the extent of binding of univalent anions over the concentration range 0.0625–0.100M, and of Na^+ over the range 0.0875–0.100M: in this connexion the results of studies on the binding of Cl^- ion [Fig. 2 of Carr (1953)] and of Na^+ ion [Table 5 of Carr (1956)]

indicate virtual absence of binding of these two ions to ovalbumin at neutral pH. However, because of these assumptions about the binding of univalent ions, the equilibrium constant obtained for the interaction of bivalent phosphate with ovalbumin can be regarded as an approximate value only: but such criticism should also be seen in the light that even approximate estimates of equilibrium constants are extremely difficult to determine for such weak ligand interactions.

In summary, an electrophoretic method has been developed for the quantitative measurement of interactions of ions with proteins, and illustrated with the ovalbumin–phosphate system at pH6.1. The procedure, which is considerably simpler to apply than its predecessor (Smith & Briggs, 1950; Alberty & Marvin, 1950; Cann, 1970), could prove of value in the detection and study of weak interactions involving ions whose biological concentrations are relatively high.

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