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ISOELECTRIC FOCUSING OF INTERACTING SYSTEMS. V. DETERMINATION OF LIGAND-BINDING CONSTANTS *

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A theory is formulated for an isoelectric focusing procedure which permits determination of intrinsic ligand-binding constants. The protein is first focused in the absence of ligand, after which ligand is added to the appropriate electrode compartment and then driven by the electric field into the focusing column where it complexes with the protein. The band of protein and its complexes moves to the constituent isoelectric point. An equation linearly relates the reciprocal of the overall distance moved to the reciprocal of the local concentration of ligand. The quotient of the intercept and slope gives the intrinsic binding constant. If the concentration of ligand in the electrode compartment is used in lieu of the local concentration, an apparent constant is obtained. Extrapolation of the apparent constant to infinite dilution of protein gives the intrinsic constant. For certain systems, conditions may be realized which give an apparent constant within 4% of the intrinsic constant.

1. Introduction

Park [1] has reported the following isoelectric focusing experiment on the preferential binding at ATP to deoxy versus CO hemoglobin: A solution of hemoglobin A was partially saturated with CO and electrofocused in an anearobic, polyacrylamide gel column containing a preformed pH gradient, pH 6–9 Ampholines. The sample focused as two distinct bands, an upper band corresponding to deoxy Hb and a lower one to the more acidic CO-Hb. Radioactive ATP was then added to the cathode compartment and electrolysis resumed so as to drive ATP down into the column. Shortly thereafter the upper band started to migrate down the column, passed through the CO-Hb band and focused at a new isoelectric point characteristic of deoxy Hb with ATP bound. After establishing that further addition of ATP caused no further migration of the band, the gel was sliced; and the ATP-deoxy Hb band, the CO-Hb band and another slice of the gel of equivalent size were eluted. The ATP concentration in the elutants was determined by radioactive counting, and the protein concentration assayed by amino acid analysis. This procedure yielded a stoichiometry of one molecule of ATP bound per tetramer. Experiments on the binding of inositol hexaphosphate to hemoglobin showed a similar migration of the deoxy Hb band, but the final isoelectric point was considerably more acidic presumably due to the greater negative charge on the ligand.

Although the stoichiometry of ATP-binding was thusly determined, the binding constant could not be, since there was no theory to guide the design and interpretation of the appropriate experiments. We have now formulated such a theory.

2. Theory

The theory consists of two parts: (1) analytical adaptation of the ligand-binding isotherm to an extension of the isoelectric focusing procedure described

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above and (2) numerical evaluation of the conditions under which the binding theory can be applied to the steady state, eventually reached in the isoelectric focusing column as a result of the continual flux of ligand through the column.

2.1. Binding theory

Consider that the electric field drives ligand, $\mathcal{L}$, out of the electrode compartment into the focusing column. It soon reaches the prefocused band of protein, $\mathcal{P}$, with which it interacts to form a number of complexes, $\mathcal{PL}$, ..., $\mathcal{PL}_i$, ..., $\mathcal{PL}_n$, where n is the maximum number of binding sites on the protein molecule. Formation of the successive complexes may be represented by the following equilibria [2,3]

$$\begin{aligned} &\mathcal{P} + \mathcal{L} \rightleftharpoons \mathcal{PL} \\ &\quad\vdots \\ &\mathcal{PL}_{i-1} + \mathcal{L} \rightleftharpoons \mathcal{PL}_i \\ &\quad\vdots \\ &\mathcal{PL}_{n-1} + \mathcal{L} \rightleftharpoons \mathcal{PL}_n \end{aligned} \qquad (I)$$

In general, the electrophoretic velocities and isoelectric points of the complexes will differ from each other and from $\mathcal{P}$, so that the band will migrate to a new position in the column. Let us consider the case of statistical binding to n equivalent sites with intrinsic binding constant K_0. We assume that (1) the rates of complex formation and dissociation and sufficiently fast that local chemical equilibrium obtains at every instant of electrofocusing; (2) the binding of ligand, while changing the net charge on the protein, has a negligible effect on its frictional coefficient; and (3) each ligand molecule bound increases the electrophoretic velocity of its complex by the same amount, w. Under these assumptions the constituent electrophoretic velocity of the protein in the equilibrium mixture of $\mathcal{P}$ and its complexes $\bar{V}_{\mathcal{P}}(x)$, as a function of position, x, in the column is [3,4]

$$\bar{V}_{\mathcal{P}}(x) = V_{\mathcal{P}}(x) + \bar{i}w \,. \qquad (1)$$

In this equation $V_{\mathcal{P}}(x)$ is the velocity of $\mathcal{P}$, and $\bar{i}$ is the number of moles of $\mathcal{L}$ bound per mole of total protein as given by the ligand-binding isotherm [2]

$$\bar{i} = n K_0 C_{\mathcal{L}}(x)/[1 + K_0 C_{\mathcal{L}}(x)] \,, \qquad (2)$$

in which $C_{\mathcal{L}}(x)$ is the local equilibrium concentration of free $\mathcal{L}$. Experimental verification of equation (1) is provided by the pioneering electrophoretic studies of Smith and Briggs [4] on the binding of methyl orange by bovine serum albumin. They found that the constituent mobility of the protein is a linear function of $\bar{i}$ determined by the method of equilibrium dialysis, the linearity extending to the largest $\bar{i} = 20$ measured.

We now introduce the assumption that $V_{\mathcal{P}}(x)$ is a linear function of x

$$V_{\mathcal{P}}(x) = a - bx \,, \qquad (3)$$

so that eq. (1) becomes

$$\bar{V}_{\mathcal{P}}(x) = (a + \bar{i}w) - bx \,. \qquad (4)$$

Justification for this assumption is two-fold: (1) Practitioners of isoelectric focusing strive for a linear pH gradient [5–7] and (2) The electrophoretic mobility of proteins generally varies linearly with pH in the region of the isoelectric point, the linearity sometimes extending more than 1 pH unit on either side of the isoelectric point [8–12].

By noting that $\bar{V}_{\mathcal{P}}(x) = 0$ at the position $\bar{x}$ of the constituent isoelectric point of the protein and that the position of the isoelectric point of the protein in the absence of ligand is $x_0 = a/b$, eq. (4) yields

$$\bar{x} = x_0 + \bar{i}(w/b) \,. \qquad (5)$$

Substituting eq. (2) into eq. (5) gives

$$\bar{x} = x_0 + \frac{n K_0 C_{\mathcal{L}}(\bar{x})}{1 + K_0 C_{\mathcal{L}}(\bar{x})} (w/b) \,. \qquad (6)$$

In the limit $C_{\mathcal{L}}(\bar{x}) \to \infty$, $\bar{x} \to x_f$, which is the position of the isoelectric point of the fully-liganded protein; and the constant w/b is found to be

$$w/b = (x_f - x_0)/n \,. \qquad (7)$$

Substitution of eq. (7) back into eq. (6) and rearrangement leads to the double-reciprocal relationship

$$\frac{1}{(\bar{x} - x_0)} = \frac{1}{(x_f - x_0)} + \frac{1}{(x_f - x_0)} \frac{1}{K_0} \frac{1}{C_{\mathcal{L}}(\bar{x})} \,. \qquad (8)$$

This equation permits determination of the intrinsic binding constant from isoelectric focusing experiments; but it is silent with respect to the stoichiometry of binding, because $(x_f - x_0)$ is dependent upon empirical parameters as well as n (eq. (7)).

Eq. (8) also applies to the special case $n = 1$ with a change in frictional coefficient of the protein upon

binding the ligand molecule, provided that K_0 is replaced by the apparent binding constant $K_0' = K_0(D_{\mathcal{PL}}/D_{\mathcal{P}})$, where $D_{\mathcal{PL}}$ and $D_{\mathcal{P}}$ are the diffusion coefficients of the liganded and unliganded protein, respectively. Since $D_{\mathcal{PL}}/D_{\mathcal{P}}$ usually will not differ much from 1, the difference between K_0' and K_0 will probably be within experimental error. For cooperative binding without significant change in frictional coefficient, $C_{\mathcal{L}}(\bar{x})$ in eq. (8) is replaced by $C_{\mathcal{L}}(\bar{x})^m$ where m is the Hill coefficient, and K_0 becomes an apparent binding constant. For binding to a large number of noninteracting sites with different intrinsic binding constants, we suppose a Sip's distribution of intrinsic binding affinities, in which case $K_0 C_{\mathcal{L}}(\bar{x})$ in eq. (8) is replaced by $[\bar{K}_0 C_{\mathcal{L}}(\bar{x})]^{\alpha}$ where $\bar{K}_0$ is an average intrinsic binding constant and α is Sip's heterogeneity index.

An experimental design for application of eq. (8) is as follows: (1) Focus the same amount of protein in each of a series of gel columns and record the position x_0 of the band; (2) Add differing concentration of ligand to the appropriate electrode compartment of each gel (cathode compartment for anions); and (3) Resume electrolysis until the steady state is established, the band of protein and its complexes with ligand having migrated by this time to the position $\bar{x}$. The value of K_0 is given by the quotient of the intercept and slope of the plot of $1/(\bar{x} - x_0)$ against $1/C_{\mathcal{L}}(\bar{x})$. The question immediately arises as to what value to use for $C_{\mathcal{L}}(\bar{x})$, since the concentration of free ligand in the protein-complex band would be difficult to determine. We propose that the initial concentration of ligand in the electrode compartment, C, be used [‡1]. The numerical transport calculations described below were made in order to evaluate the conditions under which this approximation can give accurate K_0-values.

2.2. Numerical transport calculations

The calculations are for the reaction

$$\mathcal{P} + \mathcal{L} \rightleftharpoons \mathcal{PL}, \quad K_0 , \qquad \text{(II)}$$

in which the protein binds a single molecule of a negatively charged ligand. Binding of the ligand causes a change in electrophoretic velocity and isoelectric point but not frictional coefficient of the protein. Transient isoelectric focusing patterns have been computed by numerical solution of the simultaneous transport equations and mass action expressions as a function of time, thereby constructing the approach to the steady state pattern. The calculations are essentially as described previously [13] except for the following modifications: (1) The appropriate mass action expressions are used for recalculation of chemical equilibrium after each cycle of diffusion and driven transport. (2) The initial condition for $\mathcal{P}$ is a gaussian distribution of concentration about the position of its isoelectric point in the electrofocusing column with maximum concentration, $C_{\mathcal{P}}^0$, and variance $\sigma^2 = D_{\mathcal{P}}/b$; i.e., a prefocused band of $\mathcal{P}$. Initially, there is no $\mathcal{L}$ in the column, only a 0.01 cm thick zone of concentration C at the cathode end representing the interface between the column and the ligand-containing electrode compartment. (3).The boundary conditions for $\mathcal{P}$ and $\mathcal{PL}$ correspond to reflection of the molecules at the ends of the column

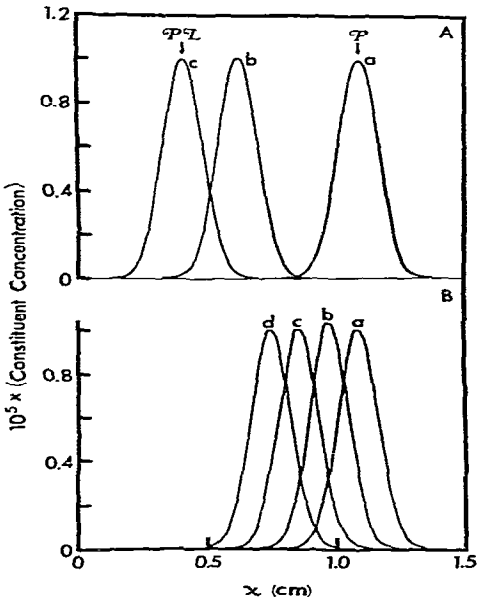

Fig. 1. Theoretical isoelectric focusing patterns illustrating the time-course of approach to the steady state (reaction (II) with $K_0 = 5 \times 10^4\ M^{-1}$ and $C_{\mathcal{P}}^0 = 1 \times 10^{-5}$ M). A–C = 1×10^{-2} M: a, $t = 0$; b, 1.2×10^4 s; c, 1.2×10^5 s. B–C = 2×10^{-5} M: a, $t = 0$; b, 4.8×10^3 s; c, 1.2×10^4 s; d, 1.2×10^5 s.

‡1 Alternatively, one may have to use the concentration in a slice of gel between the peak of constituent protein and the top of the column.

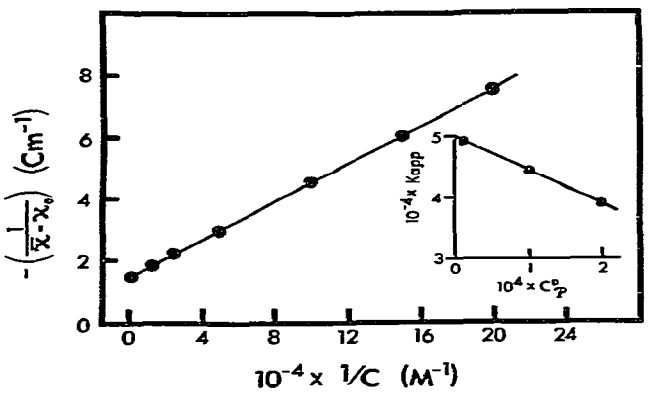

Fig. 2. Double reciprocal plot of steady-state isoelectric focusing data, •, simulated for reaction (II) with $K_0 = 5 \times 10^4$ M^{-1} and $C^0_{\mathcal{P}} = 1 \times 10^{-5}$ M. The straight line is the least-squares fit, $(\bar{x} - x_0)^{-1} = -(1.455 \pm 0.0046) - (3.031 \pm 0.0044) \times 10^{-5}\ C^{-1}$. The insert shows the extrapolation of K_{app} to infinite dilution of protein (reaction (II) with $K_0 = 5 \times 10^4\ M^{-1}$); for this purpose K_{app} was calculated from x_0, x_f, and $\bar{x}$ computed for $C = 2 \times 10^{-5}$ M.

[14]. The boundary conditions for $\mathcal{L}$ constitute a source of constant C at the cathode end of the column and a sink at the anode end; i.e., infinitely large electrode compartments.

The calculations were made on the University of Colorado CDC 6400 computer. The values of the several parameters are as follows: $\Delta x = 0.01$ cm; $\Delta t = 4$ s; $D_{\mathcal{P}} = D_{\mathcal{PL}} = 6 \times 10^{-7}$ cm^2 s^{-1}; diffusion coefficient of ligand, 1×10^{-5} cm^2 s^{-1}; driven velocity of protein species, $V_i(x) = a_i - b_i x$, with $a_i = 1.085 \times 10^{-4}$ cm s^{-1} and $b_i = 1 \times 10^{-4}$ s^{-1} for $\mathcal{P}$, and 0.405×10^{-4} cm s^{-1} and 1×10^{-4} s^{-1}, respectively, for $\mathcal{PL}$; constant driven velocity of $\mathcal{L}$, $V_{\mathcal{L}} = -5 \times 10^{-4}$ cm s^{-1}.

Isoelectric focusing patterns, distributions of ligand along the focusing column and values of $\bar{x}$ have been computed for a range of values of K_0, C and $C^0_{\mathcal{P}}$. The isoelectric focusing patterns are displayed as plots of the molar constituent concentration of protein (viz, sum of the concentrations of $\mathcal{P}$ and $\mathcal{PL}$) against x with the anode to the left. Vertical arrows locate the isoelectric points of designated species. Representative steady state distributions of free ligand along the focusing column are also presented. The value of $\bar{x}$ is taken to be the mean position of the peak of constituent protein in the steady state pattern. $C_{\mathcal{P}}(x)$ and $C_{\mathcal{PL}}(x)$ are the concentrations of $\mathcal{P}$ and $\mathcal{PL}$ at x. $V_{\mathcal{PL}}(x)$ is is the driven velocity of $\mathcal{PL}$.

3. Results

Representative isoelectric focusing patterns calculated for reaction (II) are displayed in fig. 1, which illustrates the time-course of approach to the steady state [‡2]. When the cathode compartment contains a saturating concentration of ligand (fig. 1A) the steady-state concentration of free ligand along the column is uniform and equal to C; the protein is fully liganded; and the steady-state pattern shows the peak of $\mathcal{PL}$ focused at its isoelectric point. For half-saturating concentration of ligand, $C = 1/K_0$, the peak of constituent protein in the steady-state pattern (curve d in fig. 1B) is focused virtually midway between the isoelectric points of $\mathcal{P}$ and $\mathcal{PL}$ (midpoint, 0.745 cm; maximum of peak, 0.745 cm; $\bar{x} = 0.747$ cm). Thus, the results of the transport calculations are in qualitative agreement with the binding theory. The quantitative test is to determine the value K_0 from the calculated values of $\bar{x}$ by application of eq. (8), to the approximation that C can be used in lieu of $C_{\mathcal{L}}(\bar{x})$.

The plot of $1/(\bar{x} - x_0)$ against $1/C$ is presented in fig. 2. The value of the apparent binding constant calculated from the slope and intercept of the linear least-squares fit of the simulated data points is $K_{app} = (4.08 \pm 0.17) \times 10^4\ M^{-1}$, which is to be compared with $K_0 = 5 \times 10^4\ M^{-1}$. The 4% discrepancy between K_{app} and K_0 is not due to numerical errors in the transport calculations, but rather to the fact that, except when C is saturating, the steady-state distribution of free ligand along the focusing column is not uniform throughout. As illustrated by curve a in fig. 3, there is a shallow trough at the position of the focused peak

[‡2] The steady state was reached in 1.6×10^5 s (sometimes 1.2×10^5 s) as judged by the criterium that the following quantities remain constant to 6 places between 1.6×10^5 and 4.3×10^5 s: constituent concentration of protein at each position in the pattern; the amount of each protein species, total ligand and free ligand in the column; and the mean position and variance of the distribution of the constituent concentration of protein and the concentration of each protein species, total ligand and free ligand. In practice the steady state would probably be attained much more rapidly because of the much higher electric field strength used in gel columns.

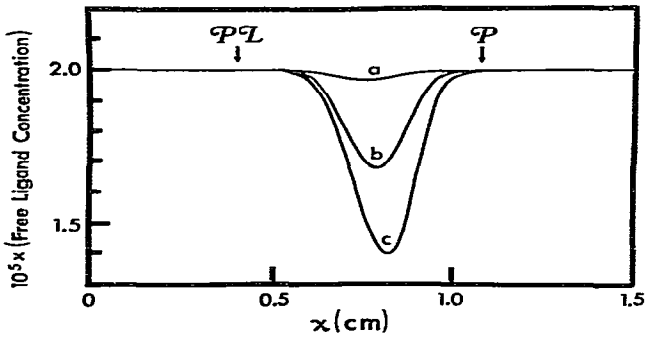

Fig. 3. Steady-state distribution of free ligand along the isoelectric focusing column. (Reaction (II) with $K_0 = 5 \times 10^4$ M^{-1} and $C = 2 \times 10^{-5}$ M): a, $C^0_{\mathcal{P}} = 1 \times 10^{-5}$ M; b, 1×10^{-4} M; c, 2×10^{-4} M.

of constituent protein so that for the value of $C^0_{\mathcal{P}}$ used in these calculations, $C_{\mathcal{L}}(\bar{x})$ is about 1.6% smaller than C. This is for $C = 1/K_0$. The discrepancy decreases with increasing C and vice versa. Accordingly, when C is used in lieu of $C_{\mathcal{L}}(\bar{x})$ in eq. (8), the strength of binding is underestimated by about 4%.

The depth of the trough in the distribution of free ligand increases with increasing $C^0_{\mathcal{P}}$ (fig. 3), as does the error in estimating the binding constant. But, as shown by the insert to fig. 2, K_0 can be accurately determined by extrapolating K_{app} to infinite dilution of protein. The same behavior was found for $K_0 = 5 \times 10^6$ M^{-1}, $C^0_{\mathcal{P}}$ ranging from 1×10^{-7} to 2×10^{-6} M.

4. Discussion

The pivotal feature of the isoelectric focusing of reaction (II) is the trough in the steady-state distribution of free ligand. We thank Dr. David J. Cox for pointing out to us that this is a necessary consequence of the law of conservation of mass. Consider the steady-state flux of constituent ligand through two transverse planes perpendicular to the isoelectric focusing column, one positioned at x_α between the focused peak of constituent protein and the top of the column and the other at $\bar{x}$. Conservation of mass requires that

$$V_{\mathcal{L}} C_{\mathcal{L}}(x_\alpha) = V_{\mathcal{L}} C_{\mathcal{L}}(\bar{x}) + V_{\mathcal{PL}}(\bar{x}) C_{\mathcal{PL}}(\bar{x}) , \qquad (9)$$

which on rearrangements gives

$$V_{\mathcal{L}} [C_{\mathcal{L}}(x_\alpha) - C_{\mathcal{L}}(\bar{x})] = V_{\mathcal{PL}}(\bar{x}) C_{\mathcal{PL}}(\bar{x}) . \qquad (10)$$

Since both $V_{\mathcal{PL}}(\bar{x})$ and $V_{\mathcal{L}}$ are negative, $C_{\mathcal{L}}(\bar{x}) < C_{\mathcal{L}}(x_\alpha)$. When $\bar{x} = x_f$, $V_{\mathcal{PL}}(x_f) = 0$ so that $C_{\mathcal{L}}(x_f) = C_{\mathcal{L}}(x_\alpha)$. As $C^0_{\mathcal{P}} \to 0$, $C_{\mathcal{PL}}(\bar{x}) \to 0$ and $C_{\mathcal{L}}(\bar{x}) \to C_{\mathcal{L}}(x_\alpha)$ so that $K_{app} \to K_0$.

The results obtained for reaction (II) are quite general and apply qualitatively to the set of reactions (I), for example. This becomes evident when the single term on the right hand side of eq. (10) is replaced by the i-weighted sum of such terms for all complexes, $\mathcal{PL}_i$, of the set. It is fundamental to the isoelectric focusing procedure elaborated here that the steady-state distribution of free ligand along the focusing column shows a trough at the position of the focused peak of constituent protein irrespective of the mechanism of binding. Moreover, mass conservation also dictates that the trough must deepen when the constituent concentration of protein is increased. Accordingly, when C is used in place of $C_{\mathcal{L}}(\bar{x})$ in eq. (8) the apparent binding constant decreases with increasing protein concentration.

The value of the binding constant can be determined with an error of only 4% or less when $C^0_{\mathcal{P}} \leqslant 1/2K_0$, which translates into a maximum permissible sample load of $97AM/K_0$ μg where A (cm^2) is the cross-sectional area of the column and M the molecular weight of the protein. For a cylindrical gel column 0.3 cm in diameter, $M = 10^5$ daltons and $K_0 = 5 \times 10^4 M^{-1}$, the maximum sample load is 14 μg. This presents no technical problem since a load of 1—4 μg per focused band is readily detectable by dye staining or ultraviolet absorption. On the other hand, when $K_0 = 5 \times 10^6 M^{-1}$ the maximum load is only 0.14 μg, and the focused band would be difficult to detect. In this case values of K_{app} must be determined at higher sample loads and extrapolated to infinite dilution to obtain K_0, but it is important to note that the extrapolation need not necessarily be linear if for no other reason than thermodynamic nonideality. While these experiments provide no information as to the stoichiometry of ligand binding, ν can be determined by the method of Park [1] as described earlier.

An advantage of isoelectric focusing is the small sample of protein required. In fact, if only a very limited quantity of material is available, K_{app} can be estimated from single determinations of x_0, x_f and $\bar{x}$ at $C \approx 1/K_0$ using the equation

$$K_{app} = \frac{1}{C}\left(\frac{\bar{x} - x_0}{x_f - \bar{x}}\right) , \qquad (11)$$

which is obtained from eq. (8). More importantly, possible heterogeneity of the material is not an impediment. As demonstrated by the experiments of Park [1] on the ATP-hemoglobin system, a band of liganded protein can pass through band(s) of protein which do not bind the ligand.

There are three limitations of the procedure: (1) The ligand must be an ion; (2) It must not be bound by Ampholine as metal ions are; and (3) Binding can not be measured at constant ionic strength maintained by strong electrolytes, but only in the milieu peculiar to isoelectric focusing.

Finally, the isoelectric focusing patterns from which the pertinent information is to be obtained, might be recorded by in situ scanning of the concentration distribution of protein in the focusing column by means ultraviolet absorption optics [15–20]. Fluorescence or refraction optics could also be used [18]. Transient state isoelectric focusing [15–19] is ideally suited to this purpose.

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