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ISOELECTRIC FOCUSING OF INTERACTING SYSTEMS.
II. PH-DEPENDENT CONFORMATIONAL TRANSITIONS*

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Transient and equilibrium isoelectric focusing patterns have been computed for pH-dependent conformational transitions in the limits of complete cooperativity and instantaneous chemical equilibration. Transitions induced by the binding of a relatively large number of hydrogen ions by the macromolecule give well resolved bimodal equilibrium patterns, provided that the resulting conformer has the lower isoelectric point. The corresponding transient patterns may be either bimodal or virtually unimodal for practical times of operation depending upon the point of insertion of the sample into the pH gradient and the stoichiometry of the interaction. A macromolecule undergoing sequential transitions can give multimodal isoelectric focussing patterns.

1. Introduction

In the first paper of this series [1] a theory of isoelectric focusing is formulated for rapidly equilibrating, carrier ampholyte-induced macromolecular isomerization. The calculations show that such systems can give well-resolved, bimodal isoelectric focusing patterns. Resolution will occur when cooperative interaction of the macromolecule with a steady-state gradient of ampholyte tends to stabilize the isomers in the vicinity of their respective isoelectric points. An analogous behavior might be expected for the rapidly equilibrating, pH-dependent conformational transition

$$\mathcal{M} + n\mathcal{H}^+ \rightleftharpoons \mathcal{N}\mathcal{H}_n^+, \quad K, \qquad \text{(I)}$$

where $\mathcal{M}$ is an amphoteric macromolecule, and $\mathcal{N}$ is its conformer with different isoelectric point (pI) formed via cooperative binding of n hydrogen ions. For this interaction, however, the value of n would have to be much larger than for ampholyte-induced isomerization, because the gradient of $\mathcal{H}^+$ along the isoelectric focusing column is shallow compared to the gradients of ampholyte. The calculations presented below bear out these expectations. They also show that the set of sequential transitions

$$\mathcal{M} + n\mathcal{H}^+ \rightleftharpoons \mathcal{N}\mathcal{H}_n^+, \quad K_1,$$

$$\mathcal{N}\mathcal{H}_n^+ + m\mathcal{H}^+ \rightleftharpoons \mathcal{P}\mathcal{H}_{n+m}, \quad K_2, \qquad \text{(II)}$$

in which $\mathcal{M}$, $\mathcal{N}$, and $\mathcal{P}$ are conformers with different pI's, can give isoelectric focusing patterns showing three peaks.

2. Theory

At the outset let us note that, since the conformers $\mathcal{M}$, $\mathcal{N}$ and $\mathcal{P}$ have different pI's due to different pK's of ionizable groups on the macromolecule, the "equilibrium constants" K, K_1 and K_2 are in general functions of hydrogen ion concentration, $C_{\mathcal{H}}$. This is true because they incorporate the pK's of the conformers in a factor, which we assume to be an insensitive function of $C_{\mathcal{H}}$. To this approximation

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the apparent equilibrium constant, defined to be the ratio of the concentrations of the conformers involved in the reaction, is equal to a constant multiplied by $C^n_{\mathcal{H}}$. This approximation becomes better as n increases because the factor, $C^n_{\mathcal{H}}$, dominates the hydrogen-ion dependency of the apparent equilibrium constant given the shallow pH gradient and the relatively small difference in pI's.

Transient and equilibrium isoelectric focusing patterns were computed for reaction (I) essentially as described for ampholyte-induced isomerization [1] except that

$$K'(x) = K[C_{\mathcal{H}}(x)]^n$$

and

$$K'(j) = K[\overline{C}_{\mathcal{H}}(j)]^n,$$

$$\overline{C}_{\mathcal{H}}(j) = (1/\Delta x) \int_{x_{j-1}}^{x_j} \exp[-(\ln 10)\mathrm{pH}(x)]\,\mathrm{d}x,$$

with $K'(j) \equiv 1$ at j midway between the two segments in which the pI's of the conformers are located. $\overline{C}_{\mathcal{H}}(j)$ is the average molar concentration of hydrogen ion in segment j. It is assumed that the buffering capacity of the carrier ampholytes is sufficient to maintain a linear pH gradient invariant in time. Values of the several parameters (including those defining the pH gradient) are the same as assigned previously, and computed patterns are displayed as plots of constituent concentration of macromolecule, C, against position, x.

Although the calculations are for a range of pH values in the region of neutrality, the results can be applied to any other range merely by translating the patterns along the pH gradient. This is so because of the linearity of the pH gradient and the way in which the apparent equilibrium constant is normalized; stated succinctly

$$K'(x) = \exp[-(\ln 10)n(\mathrm{pH}-\mathrm{pH}_{\mathrm{norm}})],$$

where $\mathrm{pH}_{\mathrm{norm}}$ is the pH at which $K'(x)$ is normalized.

Eq. (16) of Cann and Stimpson [1] was adapted to the calculation of equilibrium patterns for reaction set (II) in a 2 cm long column, for which system

$$\begin{aligned}\lambda(y) = {} & \frac{V_1(y) + V_2(y)K'_1(y) + V_3(y)K'_1(y)K'_2(y)}{D_1 + D_2K'_1(y) + D_3K'_1(y)K'_2(y)} \\ & - [(D_1 - D_2)nK'_1(y) + (D_1 - D_3)(n+m) \\ & \times K'_1(y)K'_2(y) \\ & + (D_2 - D_3)mK'^2_1(y)K'_2(y)] \ln 10(\mathrm{dpH}/\mathrm{d}x) \\ & \times \{[D_1 + D_2K'_1(y) + D_3K'_1(y)K'_2(y)] \\ & \times [1 + K'_1(y) + K'_1(y)K'_2(y)]\}^{-1},\end{aligned}$$

in which the subscripts 1, 2 and 3 denote $\mathcal{M}$, $\mathcal{NH}^+_n$ and $\mathcal{PH}^+_{n+m}$. The pI's of $\mathcal{M}$, $\mathcal{NH}^+_n$ and $\mathcal{PH}^+_{n+m}$ decrease in this order. The apparent equilibrium constant $K'_1(x) = K_1[C_{\mathcal{H}}(x)]^n = \hat{C}_2/\hat{C}_1$ is normalized midway between the positions of the pI's of $\mathcal{M}$ and $\mathcal{NH}^+_n$; $K'_2(x) = K_2[C_{\mathcal{H}}(x)]^m = \hat{C}_3/\hat{C}_2$ is normalized midway between the pI's of $\mathcal{NH}^+_n$ and $\mathcal{PH}^+_{n+m}$. The parameters defining the pH gradient are the same as above. In the case of the velocity gradients, $a_1 = 1.4975 \times 10^{-4}$ cm s^{-1}, $a_2 = 0.9975 \times 10^{-4}$ cm s^{-1}, $a_3 = 0.4975 \times 10^{-4}$ cm s^{-1} and $b_1 = b_2 = b_3 = 1 \times 10^{-4}$ s^{-1} corresponding to $\mathrm{pI}_1 = 7.50$ located at $x = 1.4975$ cm, $\mathrm{pI}_2 = 7.25$ at 0.9975 cm, and $\mathrm{pI}_3 = 7.00$ at $x = 0.4975$ cm. $D_1 = 3.6 \times 10^{-7}$ cm^2 s^{-1}, $D_2 = 3.4 \times 10^{-7}$ cm^2 s^{-1}, $D_3 = 3.2 \times 10^{-7}$ cm^2 s^{-1}.

3. Results

3.1. Reaction (I)

Pilot calculations showed that, when the pI of $\mathcal{NH}^+_n$ is higher than $\mathcal{M}$, the isoelectric focusing pattern will consist of a single peak positioned midway between the pI's where $K'(j) \equiv 1$. This is so because mass action favors $\mathcal{M}$ in the vicinity of the pI of $\mathcal{NH}^+_n$ and vice versa. On the other hand, resolution of the pattern into two peaks can occur when the pI of $\mathcal{NH}^+_n$ is lower than $\mathcal{M}$, depending upon the value of n. As illustrated by the equilibrium patterns displayed in fig. 1A, n must be fairly large ($\geqslant 8$ for the assigned pH gradient) before resolution can occur. As n is increased further, resolution improves. Indeed, for sufficiently large n ($= 30$) the pattern is reminiscent of that shown by a mixture of two noninteracting macromolecules, the two peaks being very well separated and centered at the pI's. Although each of the peaks contains only a very small amount of the other conformer ($\approx 0.02\%$ at the apex), they nevertheless constitute a reaction pattern in which the concentration of macromolecule, while very low, does not go to zero in between them. If the material in each peak

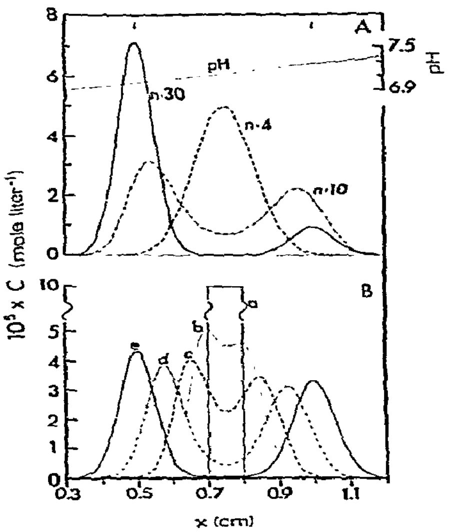

Fig. 1. Theoretical isoelectric focusing patterns calculated for the pH-dependent conformational transition given by reaction (I). A – Equilibrium patterns for indicated values of n. B – Transient patterns for $n = 30$, to be compared with corresponding equilibrium pattern presented in fig. 1A: a, $t = 0$, b, 1.25×10^3 s; c, 5×10^3 s; d, 1.25×10^4 s; e, 1×10^5 s; the amount of material in each peak changed by only ≈1.5% between 1×10^5 and 4×10^5 s. The vertical arrows in this and the following figures indicate positions of the pI's.

were to be isolated and resubjected to isoelectric focusing, the resulting patterns would also exhibit two peaks centered at the pI's.

The time-course of approach to the equilibrium pattern for $n = 30$ is presented in fig. 1B. As in the case of ampholyte-induced isomerization [1] the kinetics of approach are biphasic. Because of the exceedingly strong cooperative-uncoupling of the conformers occasioned by the large value of n and enhanced by field-uncoupling, two peaks with about the same amplitude are positioned at the pI's at a relatively rapid rate during the first kinetic phase with little opportunity for diffusional exchange of material between them. Since the concentration gradient almost vanishes midway between the pI's where the constituent velocity is zero, the second, diffusion-dominated phase proceeds at an exceedingly slow rate. The role of field-uncoupling in this process is relieved when the velocity gradients are de-

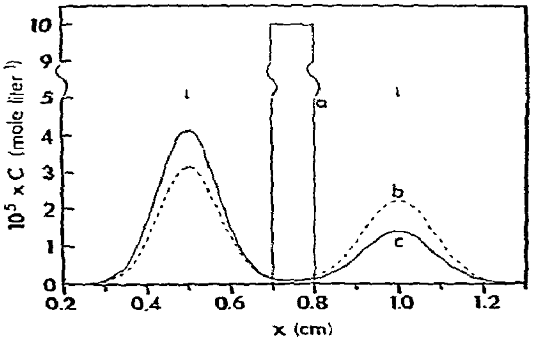

Fig. 2. Effect of halving the electric field strength on the isoelectric focusing patterns for reaction (I) with $n = 30$: a, $t = 0$; b, transient pattern at 1×10^5 s; c, equilibrium pattern.

creased by lowering the electric field strength*. Decreasing the velocity gradients increases the variance of the peaks (governed by $\bar{D}/\bar{b}$) in addition to slowing down their separation, thereby allowing for increased diffusional flow of material between peaks during both kinetic phases and, therefore, much more rapid approach to equilibrium (fig. 2). In this connection it should be noted that the smaller the diffusion coefficients (i.e., the larger the macromolecule or the smaller the pore size in gel isoelectric focusing) the smaller the variance of the peaks and, thus, the slower the approach to equilibrium. Also, the lower the velocity gradients

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[*] Lowering the electric field strength is not expected to alter the pH gradient significantly.

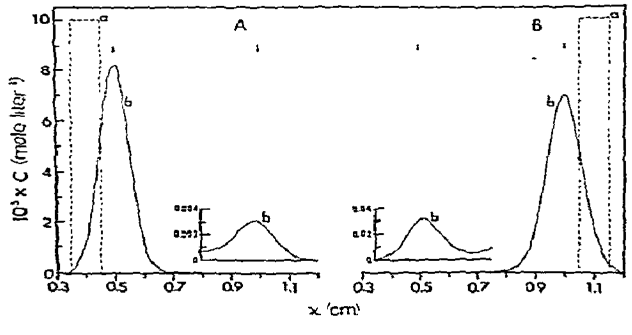

Fig. 3. Transient isoelectric focusing patterns for approach to equilibrium from an initial zone of material positioned either anodically, A, or cathodically, B, to both pI's [reaction (I) with $n = 30$]: a, $t = 0$; b, 1×10^5 s.

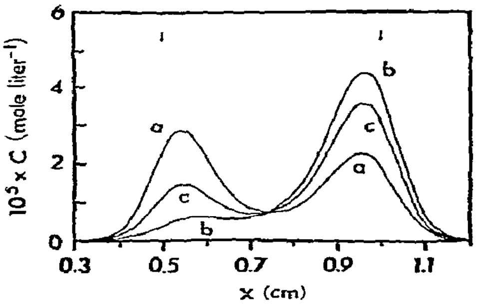

Fig. 4. Transient isoelectric focusing patterns for reaction (I) with $n = 10$: a, approach to equilibrium from an initial zone of material positioned midway between the pI's, $t = 1 \times 10^5$ s; b, initial zone positioned cathodically to both pI's, $t = 1 \times 10^5$ s; c, initial zone cathodic to both pI's, $t = 2 \times 10^5$ s.

or the greater the difference between the diffusion coefficients of the conformers other things held constant, the greater will be the disparity in peak size at equilibrium, the peak composed predominantly of the conformer with lower diffusion coefficient being the larger.

The described behavior is for the approach to equilibrium from a narrow initial zone positioned midway between the pI's. The time-course of approach is strikingly different when the initial zone is positioned either anodically or cathodically to both pI's (fig. 3). During the first kinetic phase an intense peak is rapidly positioned at the proximal pI after which time a second peak close to the distal pI grows at an extremely slow rate. This slow rate of growth is due to the vanishingly small concentration gradient of macromolecule at the midpoint where the flow of material is diffusion controlled. The rate of growth can be increased by decreasing the electric field strength thereby increasing the variance of the peaks and, thus, the gradient of macromolecule at the midpoint. Even so, the small peak might well go undetected in practice.

It is instructive to compare these results with those obtained for $n = 10$. Because cooperative-uncoupling is not nearly as severe when $n = 10$, the equilibrium pattern is approached much more rapidly and is almost reached in 1×10^5 s when the initial zone of material is centered between the pI's (compare transient pattern in a fig. 4 with the corresponding equilibrium pattern in fig. 1A). When the initial zone is positioned cathodically to both pI's, development of the peak close to the distal pI, while relatively slow, is not impeded as it is when $n = 30$. Patterns b and c in fig. 4 show that there would be no difficulty in visualizing the peak experimentally at 1×10^5 s, and that the system is about half-way to equilibrium at 2×10^5 s.

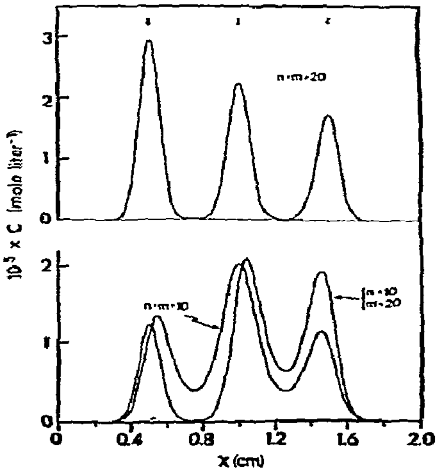

Fig. 5. Equilibrium isoelectric focusing patterns calculated for reaction set (II)

3.2. Reaction set (II)

The calculations for the set of two sequential transitions schematized by reaction set (II) show that such a system can give equilibrium patterns showing three well resolved peaks (fig. 5). Furthermore, inductive reasoning leads to the conclusion that, for a sequence of q transitions, the pattern could show $q + 1$ peaks.

4. Discussion

The calculations for reaction (I) with $n = 30$ accurately simulate the experimentally observed isoelectric focusing behavior of the 12s protein subunit of foot-and-mouth disease virus, which undergoes a pH-dependent conformational change below pH 7. Compare pattern e in fig. 1B and the patterns in fig. 3 with the experimental ones obtained by Talbot [2].

Talbot's work on this protein is a model for the study of interacting systems, particularly with respect to the way in which fractionation experiments were combined with variations in the point of insertion of the material into the pH gradient. Such experiments should be done routinely in order to distinguish between interactions and inherent heterogeneity. One question not addressed by Talbot is why the pattern shows only a single peak when the sample is focused from a point of insertion anodic or cathodic to both pI's; our calculations answer that question. They also indicate that the conformational transition is cooperative in a large number of hydrogen ions.

The isoelectric focusing of isoaccepting tRNA in the pH 3–5 range is another interacting system of some interest. Each of several highly purified isoaccepting species give patterns showing 5–8 bands with no redistribution of material between them on prolonged focusing [3]. When fractions isolated from the bands are refocused, each generates many of the bands in the original pattern. Evidence has been advanced that the bands correspond to conformers with different pI's, which are metastable at pH 8. Although binding of "Ampholines" could not be excluded, the weight of evidence suggests that the multiple forms arise from conformational transitions as the bases are protonated. Our calculations on reaction set (II) appear to furnish an explanation for resolution of the patterns. It is possible that, in the salt-free environment peculiar to isoelectric focusing, tRNA may undergo several sequential cooperative transitions in secondary and tertiary structure [4,5], each transition being induced by a moderately large number of hydrogen ions. In that event, given the appropriate parametric conditions, the equilibrium isoelectric focusing pattern would show multiple bands (fig. 5). Moreover, if the number of hydrogen ions involved in each transition is not too large, resolution of the bands would not be impeded when the sample is focused from the cathodic end of the column. In this connection we reiterate that our calculations are for the limit of instantaneous establishment of chemical equilibrium, and note that interconversion of the tRNA conformers need not be rapid. For sufficiently slow rates of reaction the diffusion barrier between the pI's is not operative during the time-course of approach to the equilibrium pattern. The concept that the constituent velocity of the macromolecule vanishes at some point between the pI's applies only in the limits where there is local chemical equilibrium among the interacting species.

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