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Classical approach to interpretation of the charge-dependence of peptide mobilities obtained by capillary zone electrophoresis

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Abstract

Published mobility measurements obtained by capillary zone electrophoresis of human growth hormone peptides are described reasonably well by the classical theoretical relationships for electrophoretic migration. This conformity between theory and experiment has rendered possible a more critical assessment of a commonly employed empirical relationship between mobility (u), net charge (z) and molecular mass (M) of peptides in capillary electrophoresis. The assumed linear dependence between u and $z/M^{2/3}$ is shown to be an approximate description of a shallow curvilinear dependence convex to the abscissa. An improved procedure for the calculation of peptide charge (valence) is also described.

Keywords: Capillary electrophoresis; Charge–mobility relationship; Electrophoretic mobility; Peptides; Growth hormones

1. Introduction

The demise of the moving boundary electrophoresis apparatus [1–3] has left capillary electrophoresis as the remaining means of measuring directly the electrophoretic mobilities of proteins and peptides. However, quantitative interpretation of mobilities obtained by the newer technique has invariably been based on generic relationships for the dependence of electrophoretic mobility (u) upon the net charge (z) borne by the peptide. Although several empirical dependencies have been postulated [4–10], the most favored expression has been the relatively simple relationship:

$$u = \frac{Kz}{M^{2/3}} \quad (1)$$

where K is a proportionality constant and M the molecular mass of the peptide. This empirical equation, which originates from analysis of peptide migration in paper electrophoresis [11], has certainly provided adequate descriptions of capillary electrophoresis data for cleavage fragments of many proteins [4,5,12–16].

One purpose of this communication is to rationalize the results for human growth hormone peptides [5] in terms of the classical expressions for migration in moving boundary electrophoresis [17,18]; and thereby to provide a means of seeking a theoretical basis for the generic expression (Eq. (1)) on which most current capillary electrophoretic analyses are based. This communication also serves to highlight inadequacies in the method by which the net charge of peptides is usually calculated; and to illustrate a theoretically more rigorous procedure [19,20].

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2. Theory

Because of the experimental design of the apparatus, the migration of a peptide in capillary zone electrophoresis is usually expressed in terms of a retention time t_P, the time taken by the peptide to migrate the fixed distance (l) from the point of sample application to the detector. However, such retention times may be converted to electrophoretic mobilities (velocity divided by electrical field strength). Specifically, the electrophoretic mobility (u) is obtained from the expression:

$$u = \frac{(l/t_P) - (l/t_M)}{E} \tag{2}$$

where the retention time t_M for a neutral marker (mesityl oxide) allows correction for the contribution of electroosmosis to the velocity of peptide migration, and E, the electrical field strength, is the applied voltage divided by the total capillary length across which the voltage is applied [5]. It is therefore of interest to determine the extent to which the mobilities thus obtained correlate with those predicted by the theory of electrophoretic migration that was developed more than 60 years ago [17,18].

2.1. Charge-dependence of electrophoretic mobility

From classical electrophoresis theory [17,18] the mobility (u) of a spherical peptide with radius r is related to its net charge (z) by:

$$u = \frac{1.6 \times 10^{-12} z f(\kappa r)(1 + \kappa r_b)}{6\pi\eta r(1 + \kappa r + \kappa r_b)} \tag{3}$$

where κ is the inverse screening length, $f(\kappa r)$ the Henry function, η the buffer viscosity, and r_b the radius of buffer ions. Obtaining the corresponding expression in terms of peptide molecular mass M entails incorporation of the relationship:

$$r = \left[\frac{3M}{4\pi\bar{v}N}\right]^{1/3} \tag{4}$$

in which $\bar{v}$ is the partial specific volume of the peptide and N the Avogadro's number [5]. It should be noted that such use of the unhydrated radius entails an assumption that the peptide is relatively unsolvated—an approximation upon which comment will be made later. Algebraic manipulation of Eqs. (3) and (4) establishes that:

$$u = \frac{F(z/M^{2/3})}{1 + [1 + \kappa r_b]/[\kappa\{3M/4\pi\bar{v}N\}^{1/3}]} \tag{5a}$$

$$F = \frac{1.6 \times 10^{-12} f(\kappa r)(1 + \kappa r_b)}{6\pi\eta\kappa[3/(4\pi\bar{v}N)]^{2/3}} \tag{5b}$$

The inverse screening length (κ) is a function of buffer conditions inasmuch as

$$\kappa = \left[\frac{8\pi N e^2 I}{1000\, DkT}\right]^{1/2} \tag{6}$$

where e denotes electronic charge, I the molar ionic strength, D the dielectric constant, k the Boltzmann constant and T the absolute temperature. Henry's function may be calculated with sufficient precision by the approximate expression [21]:

$$f(\kappa r) = 1 + \frac{0.5}{1 + \exp[2.8\{1 - \log(\kappa r)\}]}, \quad \kappa r < 10 \tag{7}$$

on the basis of the magnitude of κ and the value of r deduced from Eq. (4).

2.2. Calculation of the net charge of a peptide

Any experimental test of the predictions of Eq. (5) is clearly dependent upon the assignment of magnitudes to both M and z of the various peptides. The former may be obtained readily either by mass spectrometry or from knowledge of the amino acid sequence; but the net charge as the sum of charges on all ionizable groups is more difficult to assess. Although the fractional proton dissociation, α_i, for any given ionizable group i is in principle obtainable from the Henderson–Hasselbalch equation,

$$\mathrm{pH} = (\mathrm{p}K_{\mathrm{eff}})_i + \log\left[\frac{\alpha_i}{1 - \alpha_i}\right] \tag{8}$$

the difficulty resides in the assignment of a magnitude to the effective dissociation constant $(K_{\mathrm{eff}})_i$, which exhibits a dependence upon overall charge of the peptide [19,20]. For the situation in which all ionizable groups in any one class are intrinsically equivalent, Eq. (8) requires modification to the form:

$$\mathrm{pH} = (\mathrm{p}K_{\mathrm{int}})_i - 0.868wz + \log\left[\frac{\alpha_i}{1 - \alpha_i}\right] \tag{9a}$$

$$w = \left[\frac{e^2}{2DkTr}\right]\left[\frac{1-\kappa r}{1+\kappa r_b}\right] = \left[\frac{3.52\times10^{-8}}{r}\right]\left[\frac{1-\kappa r}{1+\kappa r_b}\right] \quad (9b)$$

where the factor $0.868wz$ takes into account the electrostatic interactions between the net charge z of the peptide at a given pH and the dissociating proton. $(pK_{int})_i$ refers to the dissociation constant for ionizable group i in a molecule with net zero charge. In the capillary electrophoresis literature only Compton and O'Grady [9] seems to have incorporated Eq. (9) into the calculation of net charge.

3. Applications of theory

Experimental data for the dependence of electrophoretic mobility upon $z/M^{2/3}$ for human growth hormone peptides (pH 2.35, 30 °C) are summarized in Fig. 1a, together with their best-fit linear description (—) reported by Rickard et al. [5]. The first task of this investigation is to compare the mobilities with those predicted by Eq. (5) in order to assess the extent to which they are describable in classical electrophoresis terms.

On the basis that the relationship between κ (cm^{-1}) and molar ionic strength I is $3.27\times10^7\sqrt{I}$ at 25 °C [18], the corresponding relationship at 30 °C is $3.28\times10^7\sqrt{I}$. Under the conditions of the experimental study ($I = 0.05$, 30 °C) [5], κ is thus $7.3\times10^6\ cm^{-1}$, whereupon the product κr ranges between 0.44 and 0.95 for $400 < M < 4000$ (the molecular mass range under consideration). Substitution of these magnitudes for κ into Eq. (7) signifies a variation of only 1.01–1.03 in $f(\kappa r)$. A fixed magnitude of 1.02 is therefore assigned to this parameter for current calculations. In keeping with practice in moving boundary electrophoresis [18,21], the radius of buffer ion, r_b, has been taken as 0.25 nm, an assignment which leads to a value of 0.18 for κr_b. After ascribing magnitudes of 0.73 ml g^{-1} to $\bar{v}$ and 0.0081 poise to the buffer viscosity (η), the value of F (Eq. (5b)) becomes 0.0258, whereupon the expression for protein mobility ($cm^2\ s^{-1}\ V^{-1}$) appropriate to Fig. 1a is:

$$u = \frac{0.0258(z/M^{2/3})}{1+(19.8/M^{1/3})} \quad (10)$$

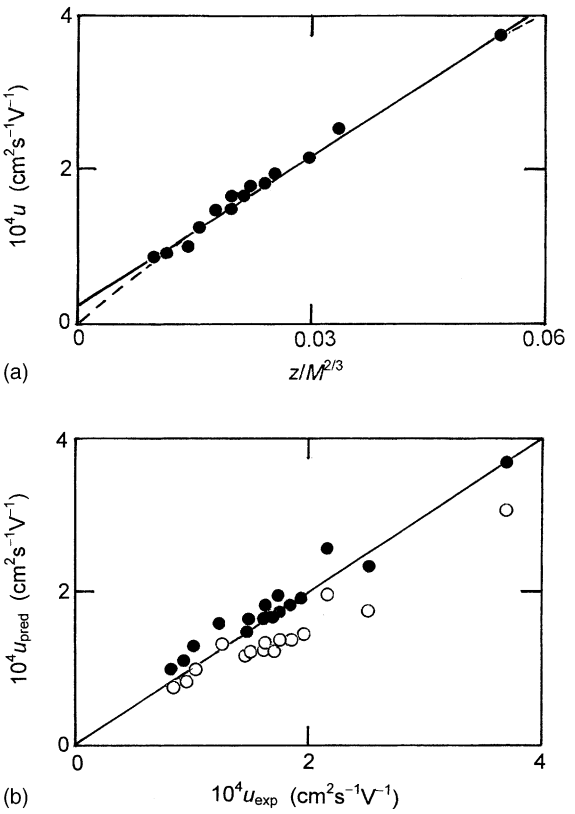

Fig. 1. Behavior of human growth hormone peptides in capillary zone electrophoresis. (a) Dependence of electrophoretic mobility upon $z/M^{2/3}$, together with its best-fit linear description (—): the broken line emphasizes the curvilinearity of the dependence. (b) Comparison of the mobilities predicted by Eq. (10) with their experimental counterparts: (●) mobilities based on the values of z used in [5]; (○) corresponding values based on estimates of z by the Skoog and Wichman [23] procedure. (Data in (a) taken from [5].)

Molecular masses for the peptide fragments of human growth hormone have been taken from Table 4 of Rickard and Nielsen [22] for calculating the final term in the denominator of Eq. (10).

The electrophoretic mobilities deduced from Eq. (10) are compared with their experimental counterparts in Fig. 1b (●), where the solid line (with a slope of unity) is the predicted correspondence for identity between theory and experiment. For mobilities greater than $1.5\times10^{-4}\ cm^2\ s^{-1}\ V^{-1}$ there is reasonable agreement between values. However, there is systematic overestimation of u_{exp} for the slowest-migrating peptides—an observation that could possibly

signify over-correction for electroosmosis, which amounts to a mobility equivalent of about $0.5 \times 10^{-4}\,cm^2\,s^{-1}\,V^{-1}$. Another potential explanation of the discrepancy between predicted and experimental mobilities for the larger polypeptides ($M \approx 2300$) is their existence in a slightly hydrated state—a factor neglected in Eq. (4) and hence Eq. (10). As noted by Compton and O'Grady [8,9], the effect of solvation can be accommodated by introducing the frictional ratio into Eqs. (4) and (5); but in the absence of experimental magnitudes for this parameter, the exercise is academic. A third potential source of the discrepancy is the value of net charge used for the calculation of $z/M^{2/3}$. Each predicted magnitude of mobility in Fig. 1b depends upon the value of z used by Rickard et al. [5]—a value that is open to criticism because of its reliance upon extents of proton dissociation calculated from Eq. (8) with $(pK_{eff})_i$ assigned the charge-independent value reported in Table 4 of [5]. In that regard the open symbols in Fig. 1b refer to predicted mobilities based on values of z deduced from another such set of effective pK values [24] for the elucidation of charge. Further clarification on this issue has therefore been sought.

As noted by Compton and O'Grady [9], the problem with calculating the extents of proton dissociation by means of Eq. (8) is that the effective pK value to be used for each class of ionizable group is a function of the net charge borne by the peptide. A preferred course of action for the calculation of net charge on a peptide thus entails the assignment of $(pK_{int})_i$ (pK in the absence of net charge) for each class of ionizable group, and allowance for the effect of net charge via Eq. (9). Because of a greater chance of group accessibility in relatively small polypeptides, the inherent disregard of group burial within hydrophobic regions of the molecule is likely to be a better approximation here than it would be for proteins [9]. Indeed, for the latter systems a major role of Eq. (9) is the detection of abnormal proton dissociation from ionizable groups [24]. Inasmuch as the use of Eq. (9) to calculate fractional proton dissociation (α_i) is conditional upon assignment of a magnitude to z, the calculation of net charge by this means is necessarily iterative. For the peptides under consideration in the present investigation this requirement has proven no great impediment in that a consistent value of z emerged after a couple of iterations for each peptide.

Table 1 summarizes the results of net charge calculations for the various human growth hormone peptides examined by Rickard et al. [5]. The calculations for peptides at pH 2.35 provide a test of the adequacy of the effective pK values assigned [5,23] to carboxyl groups, whereas those at pH 8.15 place the more stringent demands on $(pK_{eff})_i$ for the N-terminal amino group(s) as well as the histidyl and tyrosyl sidechains (there being no cysteine residues in the peptides). From the present viewpoint an important feature of Table 1 is the fairly close correspondence between z values deduced from Eq. (9) and those used by Rickard et al. [5] at pH 2.35 a finding which confirms the earlier inference that the effective pK values recommended by Skoog and Wichman [23] yield poor estimates of the extent of proton dissociation from carboxyl groups [5]. Inspection of the results of calculations for peptides at pH 8.15 signify that the disparity between estimates of z by means of Eq. (9) and the other two procedures is again greater for the Skoog and Wichman method [23]. The decision to supplant the $(pK_{eff})_i$ values reported by Skoog and Wichman [23] by another set (Table 4 of [5]) is thereby justified. However, the current method (Eq. (9)) provides an even more reliable estimate of net charge because of its allowance for the consequences of z upon $(pK_{eff})_i$—a feature absent from the Rickman et al. [5] procedure, the success of which in this particular instance can be attributed to the relatively small range of z covered by the peptides.

Having established the feasibility of employing classical theory to predict migration in capillary zone electrophoresis (Fig. 1b), we can now examine more critically the interpretation [5] placed upon the dependence of u upon $z/M^{2/3}$ (Fig. 1a). Despite the constancy of F, the prediction of a linear dependence of u upon $z/M^{2/3}$ is restricted to situations where the denominator in Eq. (10) is either effectively unity or rendered constant by an essential identity of M for all peptides. For the tripeptides ($M \approx 400$) listed in Table 1 the denominator has a magnitude of 3.7, whereas for the larger polypeptides ($M \approx 2200$) the corresponding value is about 2.5. A curvilinear plot convex to the abscissa is thus the predicted form of Fig. 1a—a conclusion supported by the experimental results when account is taken of the fact that the origin is a mandatory point for the dependence of u upon $z/M^{2/3}$ (broken line in Fig. 1a). In that regard it should also be noted that there is no unique dependence of

Table 1
Comparison of calculated net charges for human growth hormone peptides

Peptide sequence[a]	Net charge z at pH 2.35			Net charge z at pH 8.15		
	Eq. (9)[b]	[5]	[23]	Eq. (9)[b]	[5]	[23]
FPTIPSLR	1.91	1.88	1.41	0.28	0.26	0.92
LFDNAMLR	1.89	1.81	1.39	−0.63	−0.47	−0.03
AHR	2.72	2.88	2.41	0.32	0.54	0.99
LHQLAFDTYQEFEEAYIPK	2.88	2.79	2.35	−3.43	−3.47	−3.05
EQK	1.85	1.87	1.40	−0.59	−0.48	−0.03
YSFLQNPQTSLCFSESIPTPSNR | NYGLLYCFR	3.86	3.75	2.82	−0.46	−0.62	0.69
EETQQK	1.89	1.86	1.39	−1.51	−1.48	−1.03
SNLELLR	1.89	1.87	1.40	−0.62	−0.88	−0.08
ISLLLIQSWLEPVQFLR	1.93	1.87	1.40	−0.66	−0.47	−0.02
SVFANSLVYGASDSNVYDLLK	1.92	1.74	1.36	−1.66	−1.88	−1.09
DLEEGIQTLMGR	1.91	1.80	1.36	−2.49	−2.26	−2.02
LEDGSPR	1.88	1.80	1.38	−1.51	−1.47	−1.03
TGQIFK	1.87	1.88	1.41	0.28	0.52	0.89
QTYSK	1.89	1.88	1.41	0.27	0.25	0.89
FDTNSHNDDALLK	2.86	2.68	2.33	−2.35	−2.73	−2.08
KDMDKVETFLR	2.85	2.74	2.35	−0.65	−0.75	−0.13
DMDKVETFLR IVQCR | SVEGSCGF	3.84	3.75	2.81	−1.56 −1.19	−1.75 −1.35	−1.03 −0.10

[a] Taken from Table 1 of [5].
[b] Based on a temperature of 30 °C and the following $(pK_{int})_i$ values: α-carboxyl, 3.7; β,γ-carboxyl, 4.6; histidyl, 7.0; α-amino, 7.8; ε-amino, 10.3; tyrosyl, 9.6 (see Table 3 of [24]).

u upon $z/M^{2/3}$ because two peptides with different M but identical $z/M^{2/3}$ values do not exhibit the same mobility (Eq. (10)). These considerations, which have undoubtedly been responsible for the array of empirical dependencies proposed to account for the u–z–M inter-relationship [4–10], also show that the use of a plot such as that shown in Fig. 1a can only yield an approximate estimate of net charge. Fortunately, a better estimate of net charge can be obtained directly by means of Eq. (5) or (for the human growth hormone system under the present conditions) Eq. (10).

In capillary electrophoresis the emphasis has been placed on development of empirical dependencies that describe the mobility in terms of a calculated net charge [4–10]. On the grounds that the method provides a reliable measure of u, a more rewarding application of capillary zone electrophoresis should be its use to define the effective net charge. Such use of mobility data to determine the actual net charge is of particular importance in studies of proteins, where attempts to calculate z from the amino acid sequence/composition are of little merit because of their failure to take into account (i) the binding of ions other than protons and hydroxyls, and (ii) group inaccessibility arising from burial or involvement in non-covalent interactions responsible for the ternary/quaternary structure of the protein. Clearly, the direct measurement of r as a Stokes radius (instead of its elimination via Eq. (4)) allows much greater reliability to be ascribed to the net charge thus determined, because z then becomes the only parameter of unknown magnitude in Eq. (3). Although capillary electrophoresis has been used for the estimation of net charge from the effects of chemical derivatization [25] and mutation [26] upon the mobility of the protein, the inherent requirement that the modified and native forms of the protein all possess the same molecular dimensions in the solvated state is an unjustified assumption/approximation in the absence of experimental proof.

4. Summary

This investigation has served several roles. (i) It has established that the mobilities of peptides in capillary zone electrophoresis conform reasonably well with classical theory of electrophoretic migration [17,18]. (ii) It has drawn attention to potential pitfalls in the calculation of net peptide charge by current procedures [5,23], and has recommended the use of an alternative method [9,19,20,24] that takes into account the dependence of the pK for proton dissociation upon net charge. (iii) It has shown that the widely accepted linear dependence of electrophoretic mobility upon $z/M^{2/3}$ is an approximate description of a curvilinear dependence convex to the abscissa; and that the estimates of net charge by such means are only approximate. However, the essential conformity of zonal electrophoretic data with description in terms of classical theory opens up the prospect of direct determination of net charge directly from mobility measurements via Eq. (3). May the present communication encourage further studies of this potential of capillary zone electrophoresis for determining the net charge (valence) of peptides and proteins.

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