Journal of Chromatography, 173 (1979) 1–5
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 11,700

CHARGE-STATE AND CHARGE-CONTINUUM MODELS IN ELECTROPHORESIS AND ISOELECTRIC FOCUSING OF GENETIC VARIANTS

PIER GIORGIO RIGHETTI
Department of Biochemistry, University of Milan, Via Celoria 2, Milan 20133 (Italy)
(Received January 5th, 1979)

SUMMARY

A model which combines the charge-state and charge-continuum models is considered in terms of the pH at which the electrophoretic analysis is made. "Unit" charge changes are obtained when the mutated charged group is at least 2 pH units apart from the p*I* of the protein. "Fractional" charge changes are found when the differences between the p*K* of the mutated charged group and the p*I* of the protein becomes progressively smaller, or when the p*K* of the ionizable group lies on the "wrong" side of the protein p*I*. It is possible to measure the p*K* of a modified group in a protein when the Δp*I* per protonic unit in a given family of proteins (*e.g.*, haemoglobin mutants) is less than unity.

Different neutral models have been proposed to account for the observed enzyme polymorphism in natural populations[1–5]. One such model, the charge-state or ladder-rung model[2], has been adopted as a key for the interpretation of available electrophoretic data. This model assumes that the only amino acid substitutions detectable by electrophoresis would involve unit charge changes, *i.e.*, substitution of a charged for an uncharged residue and *vice versa*. The model was then extended to include also mutations of two-charge changes, such as the cases in which an acidic amino acid is mutated with a basic amino acid (*e.g.*, haemoglobins C and E)[5]. Thus these mutants would be distributed into charge classes evenly spaced in electrophoretic mobility, because unit charge changes would be reflected as unit jumps in mobility.

Johnson[6] has challenged this on the grounds that no two charged group substitutions should result in identical net charge changes. He argued that, rather than discrete charge classes, there would be a continuum of charged variants, reflecting both conformational transitions introduced by the mutation and p*K* alterations of charged groups in the neighbourhood of the mutation due to electrostatic effects and/or intramolecular hydrogen bonding affecting the degree of ionization of charged groups. Recently, Ramshaw and Eanes[7], using as a model esterase variants at the *Est-5* locus of *Drosophila pseudoobscura*, have demonstrated the charge-state model to be the most likely, no continuum of charge change being detectable, as claimed by Johnson[6].

At first sight, the above might appear to be simply a theoretical discussion. If

one positive or negative charge is added or removed from a protein (*i.e.*, if a proton is lost or acquired by a protein) it seems to be obvious that these changes will be reflected as unit jumps in mobility. However, several lines of evidence suggest that this is not often the case. I present here arguments which suggest that each model, taken singularly, is incorrect and a more realistic model is a combination of the two. Ramshaw and Eanes[7] have divided the 22 *Est-5* variants into five charge classes (+2, +1, 0, −1, −2) ranging in p*I* from 5.14 (+2) to 5.00 (−2). In going from charge state +2 to +1 (either by loss of a basic residue or by gain of an acidic amino acid) the p*I* difference (Δp*I*) between the two species is 0.05 pH unit. If the charge-state model were correct, this Δp*I* in isoelectric focusing should be constant from one to the other charge class, as would be the relative mobility jumps in electrophoresis. This is expressed in Fig. 1 as a theoretical straight line of constant Δp*I* per constant charge classe jump. However, when the experimental data given by Ramshaw and Eanes in their Fig. 1 and Table I are plotted, it can be seen that the Δp*I* progressively decreases as the p*I* of the species in each charge state decreases. Thus, this line starts on its left side with the Ohta and Kimura[2] model, but ends, if extended on its right side, with the Johnson[6] model. In fact, given the slope of the experimental line, it can be safely assumed that, if the p*I* of the esterases were to be progressively lowered by addition of negative charges (*e.g.*, by deamidation of glutamine residues), the Δp*I* would become progressively smaller, down to a point of vanishing charge difference, as predicted by the Johnson[6] model.

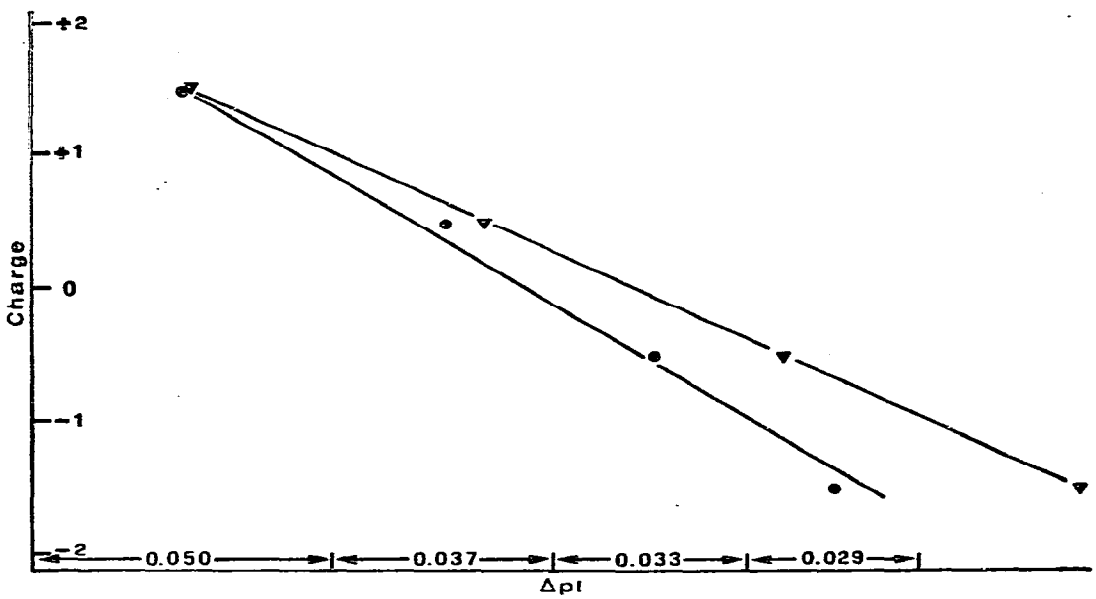

Fig. 1. Plot of charge *versus* Δp*I* in *Est-5* variants. ▲, Theoretical line of constant p*I* decrements per charge class assuming a Δp*I* of 0.05 pH unit; ●, experimental line derived from the p*I* decrements per charge class reported by Ramshaw and Eanes[7] in their Fig. 1 and Table I.

In Fig. 2 I have depicted the most common cases in which the Johnson model[6] would apply. It is odd that this should be particularly true when using isoelectric focusing which is, at present, the electrophoretic technique exhibiting the highest

resolving power. For instance, if we start with a rather basic protein (p*I* 10.5, Fig. 2A) and progressively block Lys residues, by either carbamylation (loss of a positive charge) or maleylation (replacement of a positive with a negative charge), the p*I* decrements at each step of substitution are very small at the beginning, becoming progressively larger until they attain a constant value below a given pH value. As free Lys has a p*K* of 10.53, its chemical modification will not involve "unit" but "fractional" charge changes in a basic protein. As the p*I* of the protein is lowered to *ca.* 9, each subsequent Lys modification will result in a "unit" charge change. The curve I have drawn is not just theoretical, as I have re-drawn it from the experimental data of Bobb[8] on the maleylation of chymotrypsinogen A (p*I* 9.6). By the same token, a modification of one (or more) Lys residues on a histone molecule would almost certainly be undetected in isoelectric focusing. In fact, correctly, O'Farrell *et al.*[9] have separated histones during the transient state of isoelectric focusing, in a pH 7–10 gradient, while they were still migrating electrophoretically and far from their p*I* values, where relative charge differences were maximized.

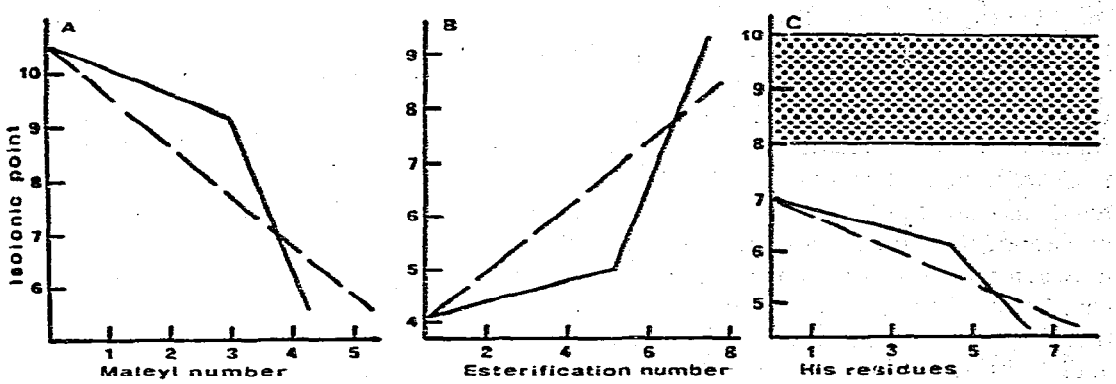

Fig. 2. The Johnson[6] and Ohta and Kimura[2] models as applied to isoelectric focusing. In all instances the broken line represents the Ohta and Kimura model and the first part of the two solid lines the Johnson model. A represents p*I* decrements upon removal of positive charges (Lys) in a basic protein (p*I* 10.5); B represents p*I* increments upon removal of negative charges (Glu or Asp) in an acidic protein (p*I* 4.0); C represents p*I* decrements upon removal of His residues in a neutral protein (p*I* 7.0). If His is removed from a basic protein (p*I* 8–10, dotted area) no p*I* decrements should be observed. The first part of the two solid lines has been drawn as a straight line only for simplicity. In reality it should be a convex exponential in A and C and concave in B.

In Fig. 2B the same situation is depicted for an acidic protein (p*I* 4) which progressively loses negative charges (*e.g.*, by esterification of Glu and/or Asp residues). As the p*K* values of side carboxyl groups in free Glu and Asp are 4.25 and 3.86, respectively, their modification again results in a "fractional" charge change, which becomes "unit" only after the p*I* of the protein has been increased above pH 5. This situation is found in the focusing of α_1-antitrypsin (P_t system). To date, 26 allele products and only 46 phenotypes (with p*I* values between pH 4.3 and 4.8) have been reported with some of the variants being so close that the separation had to be improved by resorting to focusing over a very long electrophoretic path (25 cm), by using very narrow pH ranges (covering 0.5–1 pH unit) and high voltages (up to

150 V/cm)[10,11]. By this method, the resolution limit was further improved from 0.02 to 0.005 pH unit in p*I* differences among two protein species[10].

In Fig. 2C the progressive p*I* decrements of a neutral protein (*e.g.*, haemoglobin) upon modification of His residues, or of $-NH_2$ terminal amino acids, are plotted. The situation is even worse if the same residues are blocked in basic proteins, having p*I* values in the pH range 8–10. In this instance, as shown in the dotted area, successive modifications of His and/or $-NH_2$ termini would not result in any appreciable p*I* difference, in the absence of conformational transitions in the modified macromolecule.

Table I gives the p*I* values of some genetically and chemically modified haemoglobins. "Unit" charge changes are given only by Asp, Glu, Lys and Arg mutants. "Fractional" charge changes are obtained with His mutants or upon carbamylation of the $-NH_2$ termini of α- and β-chains ($\alpha_2^c\ \beta_2^c$). In this last instance, only about one quarter of a proton per modified residue will be removed at pH = p*I*. It is of interest to speculate which is the "fractional" charge limit still resolvable by isoelectric focusing. From our experience on the focusing of haemoglobins carbamylated either on the α ($\alpha_2^c\ \beta_2$) or on the β ($\alpha_2\ \beta_2^c$) chains, it appears that the resolution limit lies close to one tenth of a proton as a charge difference among two species. As in conventional zone electrophoresis resolution is achieved only among species that differ in charge by a full proton, this represents an improvement in resolving power by one order of magnitude. As a general rule, it can be stated that, when the p*K* of a charged group is at least two pH units apart from the p*I* of a protein (on the protonated side for basic and on the deprotonated side for acidic and Tyr of Cys residues), its mutation or chemical modification will bring about a "unit" charge change. As the difference between the p*K* of the charged group and the p*I* of the protein becomes progressively smaller, or if the p*K* of the ionizable group lies on the "wrong" side of the protein p*I* (*i.e.*, in the deprotonated side for basic and the protonated side for for acidic amino acids), its mutation or chemical modification will result in a "fractional" charge difference which can be vanishing small. In this instance, zone or disc electrophoresis at appropriate pH might be preferred to isoelectric focusing. Alternatively, a titration curve of a protein and its genetic mutants could be run, by performing electrophoresis perpendicular to a stationary pH 3–10 gradient generated by focused carrier ampholytes[12,13]. In this instance, it will be possible to deduce, from the shape of the respective titration curves, which charged amino acids have been substituted.

Lastly, I think it is important, when working with any given enzyme, to determine the Δp*I* per charged residue modified, because, once this value is known, p*I*

TABLE I

p*I* VALUES OF GENETICALLY AND CHEMICALLY MODIFIED HAEMOGLOBINS

Protein (pI)		*ΔpI*	*No. of residues*	*No. of charges*
HbA (7.0)	HbS ($\alpha_2\beta_2^{6Glu \rightarrow Val}$) (7.2)	0.2	2	2
HbA (7.0)	HbC ($\alpha_2\beta_2^{6Glu \rightarrow Lys}$) (7.4)	0.4	2	4
HbA (7.0)	HbE ($\alpha_2\beta_2^{26Glu \rightarrow Lys}$) (7.4)	0.4	2	4
HbA (7.0)	HbMalmö ($\alpha_2\beta_2^{97His \rightarrow Gln}$) (6.9)	0.1	2	1
HbA (7.0)	HbWood ($\alpha_2\beta_2^{97His \rightarrow Leu}$) (6.9)	0.1	2	1
HbA (7.0)	HbA_c ($\alpha_2^c\beta_2^c$) (6.9)	0.1	4	1
HbA (7.0)	$HbA_{\frac{1}{2}c}$ ($\frac{1}{2}$ carbamylated) (6.95)	0.05	2	$\frac{1}{2}$

measurements on modified proteins will give a direct indication of the number of residues affected. It has recently been stated that "a change in net charge of one protonic unit at appropriate pH will typically shift the p*I* by about 0.1 pH unit irrespective of the total number of ionizable groups on the protein"[14]. This statement is almost certainly incorrect. The only well documented system to which this rule (Δp*I* per protonic unit = 0.1) applies is haemoglobin[15]. In thyrotropins[16], Δp*I* per protonic unit is 0.15, in yeast phosphoglycerate kinase[17] it is 0.2 and in chymotrypsinogen A[8] it is 0.25 pH unit when it is modified by maleylation and as much as 0.53 pH unit when it is carbamylated. With insulin[18], the Δp*I* between control and monoacetylated derivative is 0.25 pH unit, which becomes 0.2 between the mono- and diacetylated derivatives and 0.15 between di- and triacetylated insulin. Therefore, I think the value of Δp*I* per protonic unit will have to be determined experimentally for each given macromolecule being investigated, as it probably depends on both a minimum molecular weight and a given amino acid composition, particularly with regard to the presence or absence of histidine residues. The exact measurement of Δp*I* per protonic unit will also allow the determination of the pK' of the modified group in the case in which this value is smaller than unit in a given system. Thus, with haemoglobins, the Δp*I* per proton is 0.1 pH unit in most genetic mutants, but is much smaller than that when the $-NH_2$ termini of α- or β-chains are modified. This means that not a "unit" charge (*i.e.*, a proton) but a "fractional" charge is removed. Therefore, on the bases of the well known Henderson–Hasselbach equation, pH = pK' + log[A^-]/[HA], the Δp*I* measurement will give the ratio of protonated to deprotonated species (*i.e.*, log[A^-]/[HA]) and the pK' will be derived at pH = p*I*, provided that no conformational transitions occur in the modified macromolecule.

ACKNOWLEDGEMENT

This work was supported by CNR grants CT77.01471.04 and 78.01533.06.

REFERENCES

1 M. Kimura and J. F. Crow, *Genetics*, 49 (1964) 725.
2 T. Ohta and M. Kimura, *Genet. Res.*, 22 (1973) 201.
3 J. L. King, *Genetics*, 76 (1974) 607.
4 J. L. King and T. Ohta, *Genetics*, 79 (1975) 681.
5 A. H. D. Brown, D. R. Marshall and L. Albecht, *Genet. Res.*, 25 (1975) 137.
6 G. B. Johnson, *Genetics*, 78 (1974) 771.
7 J. A. M. Ramshaw and W. G. Eanes, *Nature (London)*, 275 (1978) 68.
8 D. Bobb, *Ann. N.Y. Acad. Sci.*, 209 (1973) 225.
9 P. Z. O'Farrell, H. M. Goodman and P. H. O'Farrell, *Cell*, 12 (1977) 1133.
10 R. C. Allen, R. A. Harley and R. C. Talamo, *Amer. J. Clin. Pathol.*, 62 (1974) 732.
11 R. C. Allen, P. M. Oulla, P. Arnaud and J. S. Baumstark, in B. J. Radola and D. Graesslin (Editors), *Electrofocusing and Isotachophoresis*, Walter de Gruyter, Berlin, 1977, pp. 255–264.
12 P. G. Righetti, R. Krishnamoorthy, E. Gianazza and D. Labie, *J. Chromatogr.*, 166 (1978) 455.
13 R. Krishnamoorthy, A. Bianchi-Bosisio, D. Labie and P. G. Righetti, *FEBS Lett.*, 94 (1978) 319.
14 J. R. Cann, D. I. Stimpson and D. J. Cox, *Anal. Biochem.*, 94 (1978) 34.
15 P. G. Righetti in P. G. Righetti, C. J. Van Oss and J. W. Vanderhoff (Editors), *Electrokinetic Separation Methods*, North-Holland/Elsevier, Amsterdam, New York, 1979, Ch. 20, pp. 389–441.
16 K. M. M. Davy, J. S. Fawcett and C. J. O. R. Morris, *Biochem. J.*, 167 (1977) 279.
17 R. A. Stinson, *Biochem. J.*, 167 (1977) 65.
18 D. G. Lindsay and S. Shall, *Biochem. J.*, 121 (1971) 737.