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PROTEIN TITRATION CURVES BY COMBINED ISOELECTRIC FOCUSING–ELECTROPHORESIS WITH HEMOGLOBIN MUTANTS AS MODELS

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SUMMARY

By performing electrophoresis perpendicular to a stationary pH gradient generated by focused carrier ampholytes in a gel slab, a pictorial representation of a protein titration curve is obtained. By running a protein and its genetic mutants in parallel, the shape of the relative titration curve reveals which charged amino acid has been substituted. Within a given family of proteins, under a constant set of experimental conditions, the relative mobility at any given pH can be correlated with the number of protons lost or acquired by the protein.

INTRODUCTION

In principle, it should be possible to construct the titration curve of a protein by following its electrophoretic mobility at a series of different pH values covering a pH 3–11 range. If a protein and its genetic mutants are run in parallel in these electrophoretic systems at multiple pH's it could be possible to deduct from the shape of the respective titration curves which charged amino acids have been substituted. In practice, this would be a long and tedious job, not amenable to routine laboratory applications. Recently, however, Rosengren *et al.*[1] have described the possibility of performing a protein titration curve in a gel slab, by performing isoelectric focusing in the first dimension, followed by electrophoresis at right angles. As this method appears to possess important future applications in protein chemistry, we have tried to standardize it by application to a well known macromolecular system, *i.e.* to hemoglobin

and its genetic mutants. The shape of the titration curve was indeed as predicted on the basis of known amino acid substitutions. Moreover, by a simple extrapolation technique, it was possible to calculate at any given pH the number of protons released or bound by the macromolecule.

MATERIALS AND METHODS

The two-dimensional technique was performed as follows: a gel slab was polymerized to contain 7% acrylamide [the ratio of acrylamide to Bis in the stock solution being 25:1 (w/w)][2], 2% Ampholine in the pH range 3.5–10, 5 m*M* Asp, 5 m*M* Glu, 5 m*M* Lys and 5 m*M* Arg. The gel slab (25 × 11.5 × 0.2 cm, LKB 2117 Multiphor chamber) was cast with a narrow trough in the middle, 10 cm long, 1 mm wide and 1 mm deep, which could be filled with about 100 μl of sample. 1 *M* NaOH and 1 *M* H_3PO_4 were soaked into the cathodic and anodic filter paper strips, respectively, and the pH gradient was preformed by delivering 13 W, with an LKB constant wattage power supply, for 90 min at 4°, across the short gel side (11.5 cm). At this point, the anodic and cathodic regions were removed by slicing the gel on the inner side of the electrodic filter paper strips. New strips, still containing 1 *M* NaOH at the cathode and 1 *M* H_3PO_4 at the anode, were applied on the short gel side (reduced to 10 cm length), the trough filled with sample (usually 100 μg for each protein species) and electrophoresis perpendicular to the preformed pH gradient performed for 30 min at 700 V. Preliminary experiments demonstrated that the pH gradient remained stationary for more than 1 h. At the end of the experiment, the pH gradient was measured on two gel slices, each 1 cm wide, cut on either side at 6 cm distance from the central trough. The gel strips were cut into 20 segments, each 5 mm long, which were eluted with 300 μl of 10 m*M* KCl and read with a combined microelectrode on a digital Radiometer pH meter[3]. The remainder of the gel was then stained using a colloidal dispersion of Coomassie Brilliant Blue G-250 in 12% TCA and 1 *N* H_2SO_4. Only the protein adsorbed the dye, leaving the gel background completely clear and unstained[4,5].

RESULTS AND DISCUSSION

Fig. 1 depicts theoretical titration curves calculated for a protein and for its genetic mutants on charged amino acids. For instance, in the case of a substitution involving Lys, the two curves should meet above pH 10, where the charge differences among the two species disappear as the Lys groups are titrated, while below pH 9 the two curves should be parallel for all the length of the titration curve, down to pH 3 (Fig. 1A). Conversely, in the case of substitutions of Asp or Glu with neutral amino acids, the two curves should meet around pH 3, where β- and γ-carboxyls are protonated, while above pH 5 they should run parallel all the way to the alkaline end (Fig. 1B). In the case of a His to neutral substitution, the two curves should join just around pH 7 and move as a single front in the alkaline branch of the titration curve (Fig. 1C), while for an Arg to neutral substitution the two curves should run parallel all along the pH field, since it is not possible by IEF to obtain stable and reliable pH gradients above pH 10.5. It is also possible to predict the shape of the titration curve for substitutions involving two charge differences, *i.e.* for cases in which a basic amino acid

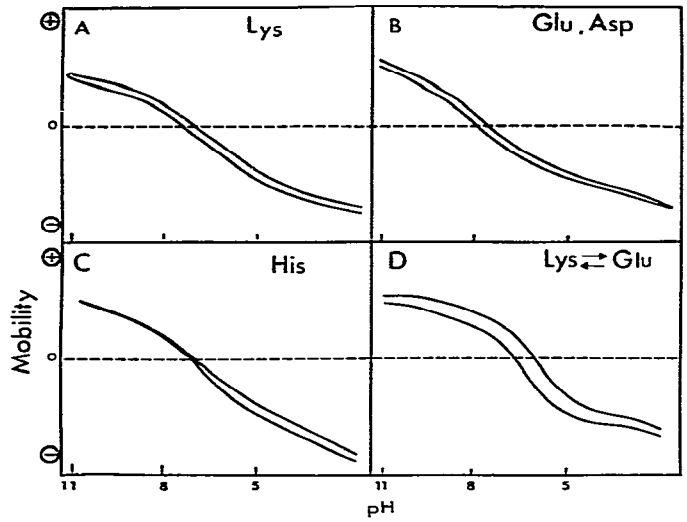

Fig. 1. Theoretical titration curves for Lys to neutral amino acid substitution (A), Glu or Asp to neutral (B), His to neutral (C) and for a double charge variant Lys ⇆ Glu (D). In each plot, one of the two curves is normal HbA and the other is the mutant Hb. The dotted line represents the zero-mobility plane of each macromolecule, *i.e.* its isoelectric plane. In the actual experiment (see Figs. 2 and 3) it represents also the application trough.

is substituted with an acidic one, or *vice versa*. A classical example is hemoglobin C (HbC: $\alpha_2\beta_2^{6(A3)\ Glu\rightarrow Lys}$). In this case, the distance between the two curves should be twice the distance found in single-charge substitution in the pH region *ca.* 5–9. As the pH is progressively decreased to 3 or increased to 11 the two curves should converge (but never meet!) to 1/2 the distance since 1/2 of the charge difference is lost at the two extremes (Fig. 1D).

Figs. 2 and 3 give the experimental validation of our theoretical titration curves. In Fig. 2A, the titration curves of HbA and HbS ($\alpha_2\beta_2^{6(A3)\ Glu\rightarrow Val}$) are seen to join at acidic pH, while they remain parallel from pH 5.5 to the extreme alkaline end. Conversely, in the case of HbA and HbG Philadelphia ($\alpha_2^{68\ (E17)\ Asn\rightarrow Lys}\beta_2$) (ref. 6), the two titration curves meet sharply above pH 10 while they stay parallel below pH 9 (Fig. 2B). In the case of the couple HbA and HbC, the two titration curves run as predicted, with twice the distance between pH 5 and 9, and 1/2 the distance outside this pH range (Fig. 3A). We have shown in Fig. 3B, the titration curve of Hb Suresnes ($\alpha_2^{141\ (HC3)\ Arg\rightarrow His}\beta_2$) (ref. 7). This curve is the reverse of the His profile of Fig. 1C, *i.e.* only one line is seen below the p*I* plane (from pH 3 to pH 7) and two above the p*I* plane (from pH *ca.* 7 to pH 10) where the additional His loses its charge. By this technique, it is also possible to distinguish the titration curves of the contaminating minor proteins present in the red blood cell lysate. In our case, two other minor proteins are visible, one with a p*I* of *ca.* 6, the other, more acidic, with a p*I* of *ca.* 5 (most probably carbonic anhydrases). While the acidic portions of the titration curves of these proteins are well away from the Hb curves, the alkaline parts run very close and even cross the Hb titration curves.

In addition to determining which charged amino acid has been substituted, it

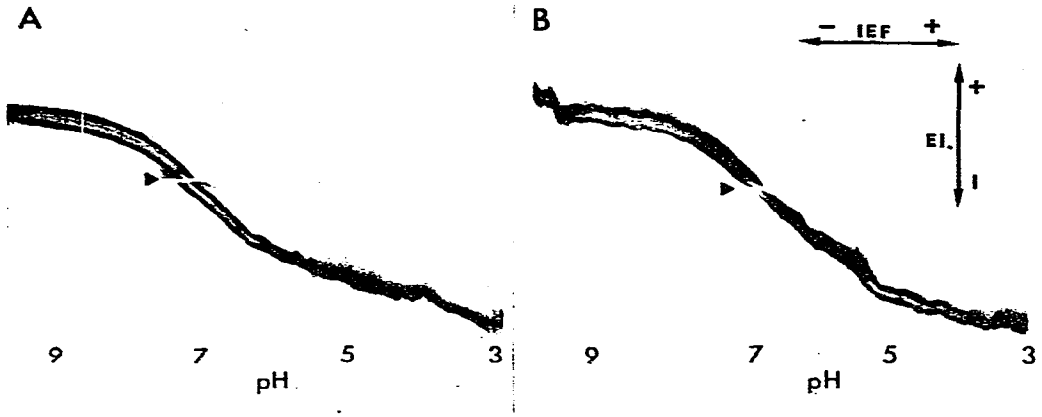

Fig. 2. Titration curves of HbA and HbS (A) and HbA–HbG Philadelphia (B). Notice also the pH–mobility curves of two other minor protein fractions, with p*I*'s of *ca.* 6 and 5, present in the red blood cell lysate. About 100 μg per each Hb species are loaded in the gel trough. The two arrows and positive and negative symbols represent the direction and polarity of isoelectric focusing (IEF) and electrophoresis (El). The isoelectric point (p*I*) of each species is the pH of the intersection point between the protein titration curve and the sample application trough.

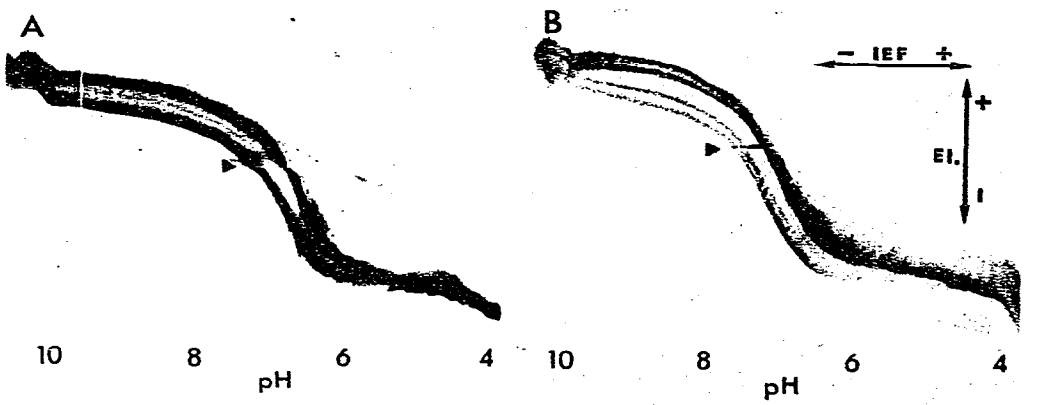

Fig. 3. Titration curves of HbA–HbC (A) and HbA–Hb Suresnes (B). The blurring of the pattern below pH 4.3 is due to decreased stability of Hb molecules. In the case of HbA–HbC (as well as with HbA–HbS and HbS–HbC) a weak band, halfway between the two titration curves, can be observed in the pH range 10 to 7. It probably represents hybrid molecules which form at the low oxygen tension in the gel and are stable only in the alkaline regions, since they disappear below the isoelectric point. In Fig. 3B the fork HbA–Hb Suresnes is exactly duplicated in the gel since the Hb samples were oxydized to Fe^{3+} forms and then reacted with CN^-. The upper p*I* fork represents *ca.* 20% met-Hb in the sample, unreacted with cyanide.

is also possible to calculate, at any given pH value, the number of protons released or bound by the protein. In our case, since HbA and HbS differ in total charge by two protons, while HbA and HbC are four protons apart, and since in the gel their relative distances are exactly proportional to the charge difference, we have assumed the migration distance from the trough to be correlated linearly with the number of pro-

tons lost or acquired by the protein. As an example, we have calculated, for HbA, HbS and HbC, that in going from their p*I* plane (7.0, 7.2 and 7.4, respectively) to pH 8.8, they differ in total charge by 12 ± 2, 10 and 8 protons, respectively. These protons will be mainly lost by His and $-NH_2$ terminal residues (and perhaps also by the two β^{93}–SH groups). This is in general agreement with the known Hb amino acid composition. The uncertainty here is due to the fact that initially not all experiments were run under identical conditions of time and voltage. However, in case of identically reproduced runs (same gel and Ampholine concentration and type, same voltage, time and temperature) the determination of the protons involved in the protein titration curve should be much more accurate.

Of course, the present method is not applicable to proteins which are unstable in some portions of the titration curve. From this point of view, even the Hb molecule is not a perfect model since its stability tends to decrease below pH 4.5, giving blurred and altered patterns, thus making the determination of carboxyl substitutions a very difficult task. In these cases, however, the variant amino acid can still be detected by denaturing the protein and running the polypeptide chains in 8 *M* urea gels[8]. In this last case the pH–mobility profiles will be closer to an absolute titration curve, as p*K* alterations of charged groups, due to neighbouring effects in the secondary to quaternary structures, will be minimized, buried charged groups will be exposed and the macromolecule will be stripped of non-covalently bound ligands. Titration curves of a protein, under native as well as denatured conditions, will however be complementary since the information obtained under these two conditions will integrate each other.

As the present technique appears to be a very valuable tool in protein chemistry we should like to stress the following general aspects:

(1) by analyzing the shape of the pH–mobility curves of a protein and of its genetic mutants, it is possible to determine which charged amino acid has been substituted;

(2) the same experiment will also allow measurements of the p*I* values of the protein of interest and of its contaminants;

(3) the titration curves of all the protein species present in a sample allow the selection of the proper purification strategy in ion-exchange chromatography, preparative electrophoresis, isoelectric focusing and isotachophoresis. As an example, it can be seen (Figs. 2 and 3) that for separation of Hb variants from the minor components of the red cell lysate, best results will be obtained in the acidic portion of the titration curve (especially in the pH range 5.5–6.5) where the proteins are widely separated while poor separations can be expected by operating in the alkaline pH region of the titration curves, especially between pH 7.5 and 8.5;

(4) titration curves can also be obtained from highly contaminated samples, for instance during the early stages of a protein purification procedure, provided the enzyme can be detected in the gel by a specific zymogram technique;

(5) a protein titration curve is indeed one of the best criteria of charge homogeneity for a protein sample. In fact if two proteins have very similar p*I* values or mobilities at a given pH, both steady-state isoelectric focusing and disc electrophoresis might fail to resolve them. On the other hand, no two dissimilar proteins, even when exhibiting very similar amino acid compositions, can possibly have exactly overlapping titration curves. Therefore, we suggest that the present two-dimensional

technique be adopted by protein chemists as an additional criterion (perhaps the most stringent) of a protein charge homogeneity.

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