CHROM. 25 251

High-efficiency capillary electrophoretic separation of basic proteins using coated capillaries and cationic buffer additives

Evaluation of protein–capillary wall interactions

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ABSTRACT

The joint use of basic cationic additives (morpholine and several tetraazamacrocycles) in the buffer and chemically bonded cross-linked polyacrylamide-coated capillaries was evaluated as a method for decreasing the adsorption of basic proteins on the fused-silica capillary wall. The superiority of the tetraazamacrocycle Cyclen (1,4,7,10-tetraazacyclododecane) over morpholine and other tetraazamacrocycles is demonstrated. Using 20 mM phosphate–60 mM Cyclen buffer (pH 5.5) and cross-linked polyacrylamide-coated capillaries, separation efficiencies in the range of 10^6 plates/m were obtained for basic proteins. A simplified model that allows the quantification of the interactions between proteins and the capillary wall was developed. The model was assessed using the different buffers and capillaries evaluated in the first part. As the model predicts, a straight line for the plot of the inverse of the migration time *versus* the electric field strength with an intercept different from zero was observed. The value of the intercept correlates with the separation efficiency observed for the basic proteins studied and, therefore, with the interaction strength between proteins and the capillary wall.

INTRODUCTION

Theoretically, more than 10^6 plates/m could be obtained in the separation of proteins using Capillary Electrophoresis (CE). However, mainly owing to adsorption of proteins on the fused-silica surface of the capillaries used in CE, the efficiency values obtained in the separation of these biopolymers is lower than that predicted by theory [1,2]. The main cause of protein adsorption on the capillary wall is the electrostatic interaction between the protein's positively charged residues and the negatively charged silanol groups intrinsic to the silica surface. In the case of proteins, the cooperative effect [3,4] could play a major role in the adsorption and the mass transfer term of the proteins during separa-

tion processes. This effect is related to the flexibility of the polypeptidic chain of the proteins, and has the result that with adsorption of the protein molecule at one site of the surface, the adsorption of this molecule at a second point of the surface is made easier or more probable.

Different methods have been developed in CE to avoid interactions between proteins and the capillary surface. These procedures include manipulation of the buffer (use of extreme pH [5,6] or the use of cationic or zwitterionic additives in the buffer [2,7,8]) or manipulation of the silica surface (see ref. 9 for an up-to-date review) to shield the active sites on the capillary wall. Each method has its advantages and drawbacks, which are summarized in ref. 9. Some of the procedures involving cationic additives in the buffer [10] and some of those using hydrophilic polymeric coatings chemically bonded to the capillary wall [11,12] have allowed the achievement of

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very high efficiencies (more than 10^6 plates/m) in protein separations.

We have demonstrated previously [13] that the joint use of cross-linked polyacrylamide-coated capillaries and buffers containing basic organic compounds (quaternary ammonium salts, linear amines, morpholine, etc.) is very efficient in decreasing the adsorption of proteins on the capillary surface. Separation of proteins using capillaries with a bonded polyacrylamide coating but without a cationic additive in the buffer gave rise to lower efficiencies than those achieved using coated capillaries with such compounds in the separation buffer. This result could be due to the fact that the polymeric layer does not cover the fused-silica surface completely or that it is not thick enough to mask the residual effect of the negatives charges on the surface [14]. We showed that the stronger the interaction between a cationic additive and surface charges, the higher are the efficiencies obtained for the separation of basic proteins. This result led us to speculate that other basic compounds able to interact more strongly with the negatively charged silanol groups of the surface could be better masking agents for the separation of basic proteins.

Tetraazamacrocycles are cyclic polyamines bearing four atoms of nitrogen in a rigid structure, several of which are completely ionized at the pH used in CE for the separation of basic proteins (pH 3–9). Their cyclic structure, where the nitrogen atoms are symmetrically distributed around the cavity whose size depends on the number of carbon atoms in the ring, may contribute more effectively to the masking effect of the silica negative charges by taking advantage of the preorganization principle observed in macrocyclic compounds [15]. The superior efficiency of these compounds as maskers of the silanophilic effect compared with other monoamine and linear polyamine compounds has been already demonstrated in HPLC [16].

The primary objective of this work was to study the effect of several tetraazamacrocyclic compounds as masking agents of the negative charges on the surface of silica capillaries used in CE.

On the other hand, it has been theoretically [1,17] and experimentally [2,5–8,12,13] demonstrated that the efficiency loss observed in the separation of proteins by CE is related to the interactions between proteins and the capillary wall. However, to the best of our knowledge, facile methods allowing the quantification of such interactions do not exist. The efficiency of the different procedures developed to reduce or preclude protein–capillary wall interactions has been evaluated so far in terms of the plate number per metre of column obtained for basic proteins separated in acidic buffers [2,5–9,13,14] or in terms of the recovery of such proteins [4].

Second, a simplified model was developed that allows the quantification of the interaction between proteins and the capillary wall. The model uses only the data obtained from the variation of the migration time of each protein with the electric field. It is easily implemented under any separation conditions (buffer and modified capillary), allowing the comparison of several methods developed to decrease the adsorption of the proteins on the capillary wall. The validity of the method was thereby established by comparing the data obtained from the model with the efficiency obtained for some basic proteins on cross-linked polyacrylamide-coated capillaries and separation buffers containing basic organic additives (morpholine or 1,4,7,10-tetraazacyclododecane) or those containing no additives.

EXPERIMENTAL

Instrumentation

Separations were carried out using a laboratory-made electrophoresis system. The apparatus included a Glassman (Whitehouse Station, NJ, USA) PS/EH50R2 power supply and a Linear Instruments (Reno, NV, USA) M-200 variable-wavelength UV–Vis detector operated at 230 nm with a laboratory-modified flow cell. The cooling of the capillaries at room temperature was achieved with a fan. Electropherograms were recorded and analysed using an A/D converter (Flytech, Taiwan), a laboratory-built amplifier, a Model 500 PC Acer computer (Multitech, Taiwan) and a PASCAL program developed in this laboratory. During electrophoresis, the current through the capillary was

measured using a Fluke (Everett, WA, USA) Model 83 multimeter. Fused-silica capillaries of 25 μm I.D. and 360 μm O.D., were obtained from Polymicro Technologies (Phoenix, AZ, USA). The total and the effective (from the injection point to the detector) lengths of each capillary used are given under Results and Discussion. The method utilized to prepare the cross-linked polyacrylamide-coated capillaries has been described previously [13]. Injection was carried out in the anode by electromigration.

All the experiments involving efficiency determination and reproducibility were accomplished in a P/ACE 2000 HPCE electrophoresis apparatus (Beckman, Fullerton, CA, USA) controlled by an IBM PS/2 286 computer. The fused-silica capillaries used in this apparatus were similar to those used in the laboratory-made apparatus, but with a 27 cm total length and 20 cm effective length. In this case, the external temperature of the capillaries was maintained at 22°C. Injection was carried out in the anode using nitrogen pressure (0.5 p.s.i.; 1 p.s.i. = 6894.76 Pa). Detection took place at 214 nm. Data were collected and analysed using System Gold software from Beckman running on the IBM PS/2 286 computer. In order to increase migration time reproducibility, the capillaries were successively rinsed for 30 s each with water, air and buffer between injections.

Samples and chemical

Lysozyme (chicken egg white) (Lys), cytochrome c (horse heart) (Cyt c), ribonuclease A (bovine pancreas) (Rib A) and α-chymotrypsinogen (bovine pancreas) (α-Chy) were purchased from Sigma (St. Louis, MO, USA) and used as received. The proteins were dissolved at the concentrations indicated (ranging from 0.2 to 1 mg ml^{-1}) in water purified using a Milli-Q system (Millipore, Bedford, MA, USA), stored at -5°C and warmed to room temperature before use. Phosphoric acid, sodium dihydrogenphosphate (both from Merck, Darmstadt, Germany), morpholine, 1,4,7,10-tetraazacyclododecane (Cyclen), 1,4,8,12-tetraazacyclopentadecane ([15]aneN$_4$) and 1,4,8,11-tetraazacyclotetradecane (Cyclam) (all from Aldrich, Steinheim, Germany) were used as received in

the preparation of the different running buffers. Hydrochloric acid, sodium hydroxide (both from Merck), 3-methacryloxypropyl-3-trimethoxysilane (ABCR, Karlsruhe, Germany), acrylamide, N,N'-methylenebisacrylamide, ammonium peroxidisulphate, and N,N,N'N'-tetramethylenediamine (TEMED) (all from Schwarz, Cleveland, OH, USA) were used for the preparation of the cross-linked polyacrylamide-coated capillaries.

Buffers

A stock solution of 20 mM phosphate buffer (pH 5.5) was prepared by dissolving the weighed amount of sodium dihydrogenphosphate in Milli-Q-purified water and adding 1 M sodium hydroxide to adjust the pH to 5.5. Aliquots of this solution were used to prepare the buffers which contained the cationic additives. The additives (morpholine or one of the three tetraazamacrocycles tested) were dissolved in the phosphate buffer at the concentrations indicated. The pH of these solutions was returned to 5.5 using concentrated phosphoric acid. The buffers were stored at 4°C and heated to room temperature before use.

Electroosmotic flow measurements

The electroosmotic flow of uncoated and coated capillaries was measured using acetone dissolved in 20 mM phosphate buffer (pH 5.5). The same buffer was used as the separation buffer for these tests. The acetone sample was injected by electromigration (10 kV, 20 s). The test was run until acetone left the column or until 3 h had elapsed.

THEORY

In electrophoresis, the main driving force for a charged particle is the electric field. If a particle p having a net positive electric charge Q_p is introduced into an electric field whose voltage gradient or electric field strength is E, a constant electric force F_e is exerted on the particle. Simultaneously, as the wall of the fused-silica capillary is negatively charged, it behaves as an electric charge Q_c, generating an electric field inside the capillary. Let us assume that this

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internal field is uniform along the capillary and, although much smaller than the external field, is not negligible and exerts a force F_i on the particle. The force F_i will have two components: the component F_{ix} directed along the capillary tube, and the component F_{iy} perpendicular to the capillary wall. The component F_{iy} could be considered as the component of F_i responsible for the adsorption of the particle on the capillary and F_{ix} as the component of F_i decreasing the migration velocity of the particle. If, as in the Hückel model [18], we suppose that the particle is very small but the double layer is thick enough to assume that the effect of viscous forces F_v is important, then

$$F_v = F_e - F_{ix} \tag{1}$$

The value of F_e is given by

$$F_e = Q_p E \tag{2}$$

and, according to the Stokes equation,

$$F_v = 6\pi\eta v_p a_p \tag{3}$$

where η is the buffer viscosity, v_p is the particle velocity and a_p its radius.

Combining eqns. 1, 2 and 3, we obtain

$$6\pi\eta v_p a_p = Q_p E - F_{ix} \tag{4}$$

The component x of the force between the capillary wall and the particle is given by

$$F_{ix} = \frac{Q_p Q_c}{\varepsilon x^2} \tag{5}$$

where ε is the dielectric constant of the buffer and x is the average distance between the surface and the particle.

By substituting eqn. 5 in eqn. 4, we obtain

$$v_p = \frac{1}{6\pi\eta a_p}\left(Q_p E - \frac{Q_p Q_c}{\varepsilon x^2}\right) \tag{6}$$

As the movement of the particle in the capillary is uniform, time t needed to move it a distance l from the injection to the detection point is given by

$$\frac{1}{t} = \frac{\mu_p}{l}\cdot E - \frac{\mu_p Q_c}{l\varepsilon x^2} \tag{7}$$

where μ_p is the electrophoretic mobility of the particle.

It can be deduced from eqn. 7 that a plot of $1/t$ against E should give a straight line whose slope depends on the electrophoretic mobility of the particle. Its intercept is also a function of both the electrophoretic mobility of the particle and the electric charge of the capillary surface. The intercept of the plot gives, therefore, a value related to the electric charge of the wall for a given particle and separation conditions. Only in those cases where the surface capillary behaves as if it were not charged ($Q_c = 0$) could the migration time be given by the classical equation
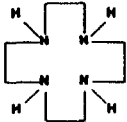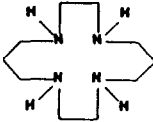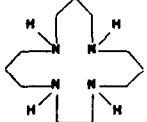
$$1/t = \mu_p E/l \tag{8}$$

For proteins, which are large non-spherical particles, moving in an electrophoretic buffer these equations must be corrected considering the effective charge and radius of the protein as a function of the buffer composition, which does not substantially modify eqn. 7. In this model, the electroosmotic flow was considered to be zero. In fact, using the cross-linked polyacrylamide coating described in this paper, the electroosmotic flow in the capillaries, if any, is at least two orders of magnitude smaller than that obtained with uncoated capillaries, as will be shown under Results and Discussion.

RESULTS AND DISCUSSION

Masking effect of tetraazamacrocycles on the silica surface negative charges

Tetraazamacrocycles are extremely basic compounds, able to interact with both inorganic and organic anions [19]. The tetraazamacrocycles evaluated in this work (Table I) can bear positive charges at the pH 5.5 used. As demonstrated [19,20] for azamacrocycles larger than those used in this work, the stability of the complex formed between these compounds and several types of anions (inorganic and organic) is related to the macrocycle charge, the size of its cavity compared with the anion size, the conformational characteristics of the anion and the hydrophobicity of the anion. It is possible that the capacity of the small-sized tetraazamacrocy-

TABLE I

PROTONATION CONSTANTS OF THE TETRAAZAMACROCYCLES USED

Compound	Formula	pK	Temperature (°C)	Conditions	Ref.
1,4,7,10-Tetraazacyclo-dodecane (Cyclen)		(1) 10.5 (2) 9.4 (3) ~1.6 (4) ~0.8	35	H_2O, 0.2 M $NaClO_4$	26
1,4,8,11-Tetraazacyclo-tetradecane (Cyclam)		(1) 11.2 (2) 10.3 (3) ~1.5 (4) ~0.8	35	H_2O, 0.2 M $NaClO_4$	26
1,4,8,12-Tetraazacyclo-pentadecane ([15]aneN_4)		(1) 11.1 (2) 10.4 (3) 5.3 (4) 3.6	25	H_2O, 0.5 M KNO_3	27

cles used in our work to mask the negative charges on the silica surface is also related to the structural characteristics of the macrocyclic compound, such as size cavity.

To explore the role of the macrocycle size in the masking capacity for silica-surface negative charges, three tetraazamacrocycles (Cyclam, Cyclen and [15]aneN$_4$) were compared in terms of the plate number obtained for several basic proteins (Lys, Cyt c, Rib A, α-Chym) separated on uncoated silica capillaries using phosphate buffer (pH 5.5). Under these very demanding separation conditions, the proteins had a net positive charge, being able to adsorb on the ionized silanol groups on the silica. The relative influence of the three tetraazamacrocycles was clearly shown. When an unused capillary was employed for the separation of the basic proteins with 20 mM phosphate buffer (not containing any tetraazamacrocycle), the proteins were not recovered from the column. This fact helped to deduce the strong interactions between proteins and silica surface. In contrast, when 50 mM [15]aneN$_4$ was added to the 20 mM phosphate buffer (pH 5.5), the four proteins migrated out of an unused capillary, but the efficiency obtained for their peaks was very poor. Better efficiency was obtained for the proteins on unused capillaries with 20 mM phosphate buffer

containing 50 mM Cyclam (pH 5.5) (Lys 1 · 10^5, Cyt c 1 · 10^5, Rib A 2.5 · 10^5 and α-Chym 2.5 · 10^5 plates/m). Finally, the best efficiency on unused, uncoated capillaries was achieved when a 20 mM phosphate buffer containing 60 mM Cyclen (pH 5.5) was used (Lys 2.5 · 10^5, Cyt c 1.3 · 10^5, Rib A 4.5 · 10^5 and α-Chym 3.6 · 10^5 plates/m). This result clearly demonstrates that Cyclen has a higher masking effect than the two other tetraazamacrocycles tested. As deduced from Table I, under our separation conditions [15]aneN$_4$ could have at least two protonated amino groups whereas Cyclam and Cyclen could have only two. The fact that the shielding effect increases in the order [15]aneN$_4$ < Cyclam < Cyclen, which is the same order as the decreasing cavity radius, shows the important role of the macrocycles size on their masking effect.

Morpholine has been used in protein separations by HPLC as a masking agent to avoid the detrimental effect of silanol groups [21]. Using this idea, we demonstrated in a previous study [13] that the use of cross-linked polyacrylamide-coated capillaries and buffers containing morpholine systematically led to better separation efficiencies for basic proteins than in those cases where the same buffer was used with uncoated fused-silica capillaries. It was therefore considered of interest to study the effect of buffers

containing tetraazamacrocycles on the separation of basic proteins with cross-linked poly-acrylamide-coated capillaries. The separation of several basic proteins using coated capillaries and a 20 mM phosphate–60 mM Cyclen buffer (pH 5.5) is shown in Fig. 1. The efficiencies obtained for the protein peaks were in the range of a 10^6 plates/meter (Lys $1 \cdot 10^6$, Cyt c $1.1 \cdot 10^6$, Rib A $0.8 \cdot 10^6$ and α-Chym $0.8 \cdot 10^6$ plates/m). Under the same separation conditions, but using a 20 mM phosphate–0.25 M morpholine buffer (pH 5.5), efficiencies around 2–3 times smaller were observed. Good reproducibility in terms of efficiency [relative standard deviation (R.S.D.) = 3% ($n = 4$)] and migration time [R.S.D. = 0.5% ($n = 4$)] were obtained with Cyclen-containing buffers. These results demonstrate that the joint use of Cyclen and cross-linked polyacrylamide-coated capillaries is a very effective method of decreasing protein–capillary wall interactions.

As speculated previously [13], the residual adsorption effect observed for the poly-acrylamide-coated capillaries could be due to the lack of a homogeneous capillary coating which could leave some areas of the fused silica un-coated, and/or to the fact that the poly-
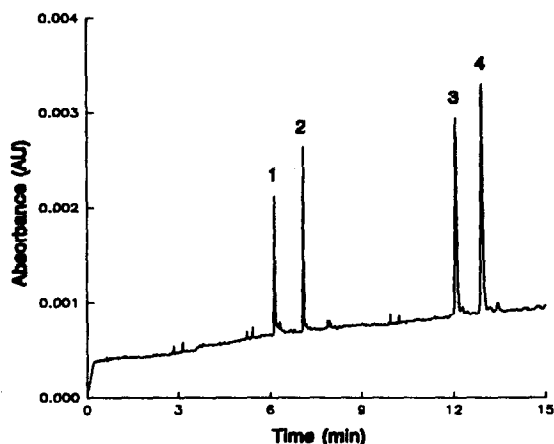
acrylamide layer, which although covering homogeneously all the silica surface, could be thin enough to let the electric charges on the silica surface adsorb the proteins on top of the polymeric layer.

In order to compare the efficiency achieved for basic proteins using polyacrylamide-coated capillaries and Cyclen-containing buffers with those predicted by theory, we used the Jorgenson's model [22], which considers that longitudinal diffusion dominates dispersion ($N = \mu VL/2Dl$). It is not easy to obtain reliable values for the diffusion coefficient (D) of the proteins in the buffers used in our experiments. However, ac-cepting that for globular proteins the Stokes–Einstein equation [23] gives a reasonable estima-tion of D, one can calculate that D values could be around $1 \cdot 10^{-6}$ cm^2 s^{-1} for Lys, Cyt c, and Rib A (molecular mass $ca.$ 15 000) and around $0.7 \cdot 10^{-6}$ cm^2 s^{-1} for α-Chym (molecular mass 25 000). Using these values and the electro-phoretic mobilities deduced from Fig. 1, an estimation gives that a maximum efficiency of $2 \cdot 10^6$–$2.5 \cdot 10^6$ plates/m should be obtained for such proteins. Several causes of band broadening in CE have been described [1], including solute adsorption, Joule heat effect, electroendosmosis and extra-column (injection and detection) ef-fects. The decrease in efficiency observed under our experimental conditions could be due to some residual adsorption of the basic proteins on the uncovered silica surface or on the poly-acrylamide coating. Further, under our ex-perimental conditions, for which no attempt had been carried out to optimize injection, this could also be a major cause of the difference in efficiency noted between theory and our results [24]. We have observed (results not shown) that the amount of protein injected (decreased by changing both the injection time and sample concentration) can dramatically increase (by more than 100%) the efficiency obtained for proteins. We are currently working on this issue.

In order to gain some practical knowledge about the effect of the concentration of basic additives on the separation efficiency and to compare Cyclen and morpholine as masking agents in CE, we compared the efficiencies obtained for several basic proteins with different

Fig. 1. Separation of basic proteins. Capillary, cross-linked polyacrylamide coated, 25 μm I.D., 360 μm O.D., 27 cm total length, 20 cm effective length; buffer, 20 mM phosphate–60 mM Cyclen (pH 5.5); temperature, 22°C; voltage, 12 kV (24 μA); injection, 2 s at 0.5 p.s.i. (nitrogen); detection, 214 nm. Peaks: 1 = 0.27 mg ml^{-1} lysozyme; 2 = 0.27 mg ml^{-1} cytochrome c; 3 = 0.35 mg ml^{-1} ribonuclease A; 4 = 0.81 mg ml^{-1} α-chymotrypsinogen.

concentrations of these additives. The results are shown in Fig. 2. In general, an increase in the concentration of the additive (Cyclen or morpholine) in the buffer led to an increase in the efficiency achieved for the basic proteins. As a general rule, better efficiencies are obtained for the same proteins separated using Cyclen-containing buffers than for those containing morpholine. It should also be noted that the optimum masking effect of Cyclen is achieved at concentrations one order of magnitude lower than that of morpholine; this result again indicates the high efficiency of Cyclen as a negative charge masker. For a more extensive comparison

Fig. 2. Effect of (A) morpholine and (B) Cyclen concentration on the separation efficiency of several basic proteins. Buffers: (A) 20 mM phosphate–morpholine (pH 5.5); (B) 20 mM phosphate–Cyclen (pH 5.5). Voltage: (A) 14 kV; (B) 12 kV. Sample: ■ = 0.27 mg ml^{-1} cytochrome c, ▲ = 0.35 mg ml^{-1} ribonuclease A; * = 0.81 mg ml^{-1} α-chymotrypsinogen; ◆ = 0.27 mg ml^{-1} lysozyme. Other separation conditions as in Fig 1.

between morpholine and Cyclen, it should be considered that the curve's general trend and efficiency values for lysozyme are similar to those obtained for cytochrome c. As indicated at Fig. 2, the efficiencies obtained for the proteins studied at a morpholine concentration higher than 0.25 M decrease slightly. This result could be due to the high electric conductivity of the buffer containing 0.5 M morpholine. Because of the large amount of morpholine in the buffer, a very large amount of phosphoric acid had to be added to the solution to return the pH to 5.5. As a result of the buffer's high conductivity, more than 3.5 W m^{-1} of power were generated in these conditions. A previous test (results not shown) with the thermostatic system used in this experiment demonstrated that it was possible to eliminate up to 2 W m^{-1} of dissipated power (for good power dissipation, the plot of voltage *versus* current values fit a straight line with a regression coefficient >0.999). This could explain why a better efficiency was obtained with buffer containing 0.25 M than with that containing 0.5 M morpholine. On the other hand, the maximum efficiency using Cyclen-containing buffers was obtained with a power dissipation of only 0.8 W m^{-1}. This is another advantage of this tetraazamacrocycle as an additive, which could be used in air-cooled electrophoretic systems where only powers as low as 1 W m^{-1} could be dissipated [25] without excessive rise of the buffer temperature and the subsequent risk of protein denaturation.

Experimental assessment of the model

The experimental results presented above form an array of separation conditions showing different efficiencies (plate number) for basic proteins depending on the additive used and its concentration in the separation buffer. Because all the data were obtained under the same experimental conditions, they show the correlation between efficiency and wall interaction for proteins. They were therefore used for a preliminary evaluation of the migration model presented in the Theory section. Two major conclusions can be drawn from this model. First, the representation of the inverse of the migration time (t) for a given protein against the field
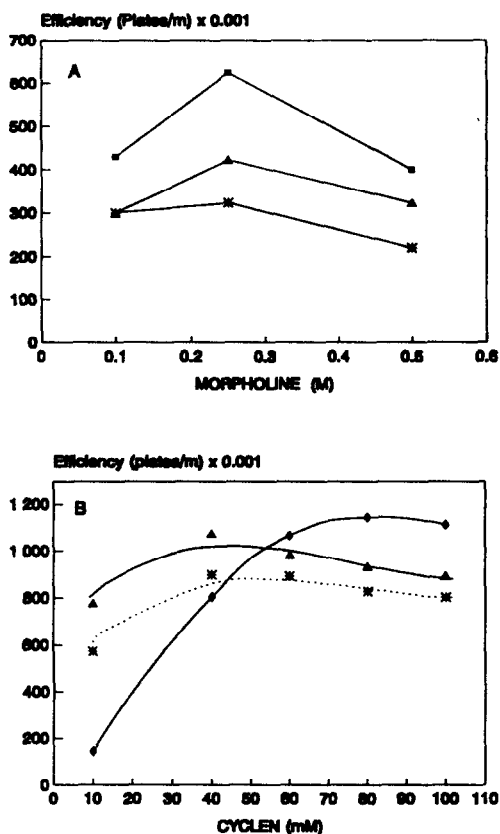
strength (E) should give a straight line with an intercept different from zero (unless $Q_c = 0$). Second, the absolute value of the intercept for each protein is related to the electrostatic interaction between the protein and the capillary wall; this value is also related to the net charge of the protein and consequently to its electrophoretic mobility under the separation conditions.

Because the model was developed assuming that no electroosmotic flow existed in the cross-linked polyacrylamide coated capillaries, we verified that the electroosmotic flow in each column used, if any, was very small. The electroosmotic flow of each capillary used in the experiments described below was measured beforehand. For the polyacrylamide-coated columns used, we observed that the electroosmotic flow coefficient (μ_{eo}) was smaller than $4 \cdot 10^{-6}$ cm^2 V^{-1} s^{-1}, that is, roughly two orders of magnitude smaller than the μ_{eo} value observed for an uncoated column tested under the same experimental conditions.

All the experiments to evaluate the model were carried out using the laboratory-made electrophoresis apparatus where the capillary was cooled using a forced-flow air system. To prevent the effect of temperature increase inside the capillary on protein migration time, special care was taken to work only under conditions (field applied and electric current) for which the power dissipated during separation was easily eliminated by the cooling system (<1 W m^{-1}).

The plot of $1/t$ for different E values using buffers containing morpholine or Cyclen for several proteins is shown in Fig. 3. As the model predicts, (eqn. 7), straight lines were obtained. The intercepts, slopes and linear regression coefficients for these straight lines are given in Table II. For a given separation buffer, the absolute values of the intercepts (A values) decrease in the order Lys > Rib A > α-Chym, that is, in the order of decreasing electrophoretic mobility. For the 60 mM Cyclen buffer, the A values for the three proteins studied are very small, and, surprisingly, they increase slightly from Lys to α-Chym (decreasing order of net charge). This can be explained in terms of a loss of statistical significance of such results if A values are compared with their standard devia-
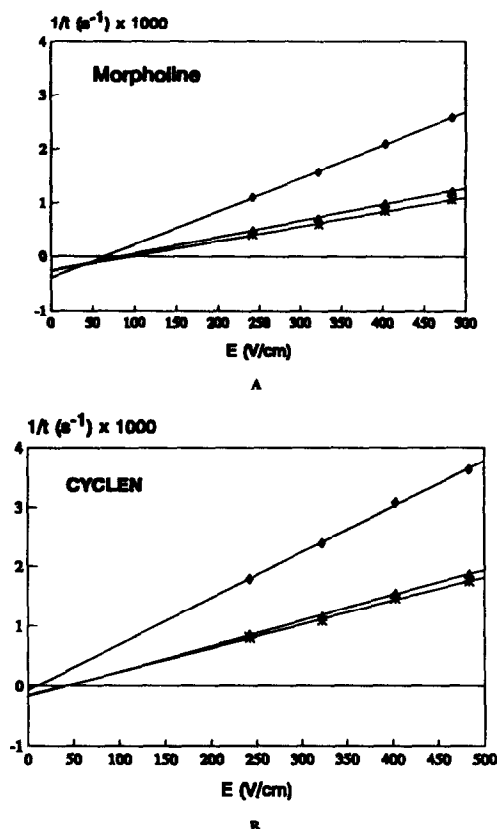

Fig. 3. Variation of $1/t$ with the field strength (E) under different conditions. Capillary, cross-linked polyacrylamide coated, 25 μm I.D., 360 μm O.D., 30 cm total length, 15 cm effective length; buffer, (A) 20 mM phosphate–0.25 M morpholine (pH 5.5) and (B) 20 mM phosphate–60 mM Cyclen (pH 5.5); room temperature; injection, electromigration, (A) 2 kV, 8 s and (B) 3 kV, 5 s; detection, 230 nm. Proteins: ◆ = lysozyme; ▲ = ribonuclease A; * = α-chymotrypsinogen.

tions (S.D.). This is related to the almost total elimination of the protein adsorption based on the high efficiency values (in the range of 10^6 plates/m) obtained for the basic proteins separated under these conditions (Figs. 1 and 2). Also, a decrease in the A values occurs for a given protein as the masking capacity of the additives increases. This effect is clearly seen when A values for no additive buffer are compared with those obtained with an additive. The same effect is observed for lysozyme on comparing the A value in 0.25 M morpholine buffer with that obtained in 60 mM Cyclen buffer. For other proteins (Rib A and α-Chym), this last

TABLE II

EFFECT OF DIFFERENT BUFFER ADDITIVES ON PROTEIN–CAPILLARY WALL INTERACTIONS

Capillary, cross-linked polyacrylamide coated, 25 μm I.D., 360 μm O.D., 30 cm total length, 15 cm effective length; buffer, no additive = 20 mM phosphate (pH 5.5); 0.25 M morpholine = 20 mM phosphate–0.25 M morpholine (pH 5.5); 60 mM Cyclen = 20 mM phosphate–60 mM Cyclen (pH 5.5); detection, 230 nm. $A \times 10^3$ = intercept of $1/t$ versus E (s^{-1}). $B \times 10^3$ = slope of $1/t$ versus E (cm V^{-1} s^{-1}). r = Linear correlation coefficient of $1/t$ versus E. Values in parentheses are standard deviations of A and B values.

Additive	Lysozyme			Ribonuclease A			α-Chymotrypsinogen		
	A	B	r	A	B	r	A	B	r
No additive	−20.7 (6.6)	131 (1.3)	0.999	−9.7 (4.0)	79 (1.3)	0.999	−7.6 (3.5)	69 (1.4)	0.999
0.25 M Morpholine	−2.8 (0.6)	56 (0.7)	0.999	−0.7 (0.2)	27 (0.6)	0.999	−0.6 (0.1)	23 (0.2)	0.999
60 mM Cyclen	−0.4 (1.6)	76 (1.4)	0.999	−0.6 (0.4)	40 (0.7)	0.999	−0.7 (0.4)	37 (0.8)	0.999

comparison is blurred by the S.D. values obtained. Finally, the slope of the straight line of $1/t$ vs. E (B values) for a given buffer decreases with decreasing electrophoretic mobility of the proteins.

Other experimental data that could be explained using the model are those relating the morpholine to Cyclen concentrations in the buffer and their masking capacity. The values for the intercept and the slope for various proteins for different morpholine and Cyclen concentrations in the buffers are given in Tables III and IV, respectively. Using any of the buffer additives evaluated in this work, the intercept of the

line decreases as the additive concentration in the buffer increases. As demonstrated above, an increase in morpholine or Cyclen concentration in the buffer, up to some value, led to a decrease in the interaction between basic proteins and the capillary wall. It should be noted that in the separation used in this experiment to obtain the plot of $1/t$ vs. E for buffer containing 0.5 M morpholine, the power dissipated was 2.9 W m^{-1}. The fact that this thermal effect considerably modifies the separation efficiency of the proteins (Fig. 2) but does not significantly modify the general trend of the A values could indicate that our model would be of some utility

TABLE III

EFFECT OF MORPHOLINE CONCENTRATION ON PROTEIN–CAPILLARY WALL INTERACTIONS

Capillary, cross-linked polyacrylamide coated, 25 μm I.D., 360 μm O.D., 50 cm total length, 25 cm effective length; buffer, 20 mM phosphate–0.1–0.5 M morpholine as indicated (pH 5.5); detection, 230 nm.

Parameter[a]	Cytochrome c			Ribonuclease A			α-Chymotrypsinogen		
	0.1 M	0.25 M	0.5 M	0.1 M	0.25 M	0.5 M	0.1 M	0.25 M	0.5 M
A	−6.6	−2.6	−1.9	−2.7	−2.1	−1.8	−1.8	−1.7	−0.3
B	8.4	5.4	3.3	5.1	3.4	2.2	4.4	3.0	1.4

[a] $A \times 10^4$ = intercept of $1/t$ versus E (s^{-1}). $B \times 10^6$ = slope of $1/t$ versus E (cm V^{-1} s^{-1}).

TABLE IV

EFFECT OF CYCLEN CONCENTRATION ON PROTEIN–CAPILLARY WALL INTERACTIONS

Capillary, cross-linked polyacrylamide coated, 25 μm I.D., 360 μm O.D., 31 cm total length, 15 cm effective length; buffer, 20 mM phosphate–10–60 mM Cyclen as indicated (pH 5.5); detection, 230 nm.

Parameter[a]	Lysozyme			Ribonuclease A			α-Chymotrypsinogen		
	10 mM	30 mM	60 mM	10 mM	30 mM	60 mM	10 mM	30 mM	60 mM
A	−32	−8.7	−0.4	−9.4	−2.7	−0.6	−6.6	−2.4	−0.7
B	120	96	75	64	50	39	56	46	37

[a] $A \times 10^3$ = intercept of $1/t$ versus E (s^{-1}). $B \times 10^3$ = slope of $1/t$ versus E (cm V^{-1} s^{-1}).

even in those instances where the small power dissipation of the cooling system could increase the temperature of the separation buffer.

ACKNOWLEDGEMENTS

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