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DETECTION OF AMPHIPHILIC PROTEINS AND PEPTIDES IN COMPLEX MIXTURES

CHARGE-SHIFT CROSSED IMMUNOELECTROPHORESIS AND TWO-DIMENSIONAL CHARGE-SHIFT ELECTROPHORESIS

SUCHARIT BHAKDI, BIRGIT BHAKDI-LEHNEN and OLE JANNIK BJERRUM

The Protein Laboratory, University of Copenhagen, 34, Sigurdsgade, DK-2200 Copenhagen N (Denmark)

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Summary

Charge-shift electrophoresis has been suggested as a simple and novel method for differentiating between amphiphilic and hydrophilic proteins (Helenius, A. and Simons, K. (1977) Proc. Natl. Acad. Sci. U.S. 74, 529—532.) This communication reports on the combination of charge-shift electrophoresis with second dimensional quantitative immunoelectrophoresis, and on a two-dimensional modification of the charge-shift electrophoresis technique. From results obtained with unfractionated human plasma proteins and human erythrocyte membrane proteins we conclude that these modifications reliably permit detection of amphiphilic proteins and peptides in complex mixtures.

Introduction

Helenius and Simons [1] have recently developed a simple technique which purportedly will rapidly disclose whether an isolated protein is amphiphilic and binds non-ionic detergent or not. Their procedure utilizes agarose gel electrophoresis of proteins in the presence of 0.5% Triton X-100 plus or minus 0.25% of the anionic detergent deoxycholate or 0.05% of the cationic detergent cetyltrimethylammoniumbromide. The latter confer their negative or positive charges at neutral pH, respectively, upon the mixed micelles they form with the surplus of Triton X-100 [2]. Binding of the micelles to amphiphilic "intrinsic" membrane protein [3—6] consequently should result in anodal (Triton X-100 + deoxycholate) or cathodal (Triton X-100 + cetyltrimethylammoniumbromide) shifts in their electrophoretic mobility, compared to their mobility in Triton X-100 alone. By contrast, charge-shifts * should not be

* In this paper, the term charge-shift will be used to denote any changes in electrophoretic migration of proteins in the presence of the charged detergents.

found if a protein is hydrophilic and does not bind non-ionic detergents. The conclusions drawn by Helenius and Simons [1] were based upon consistent data obtained with pure proteins (5 amphiphilic and 17 hydrophilic proteins). Due to the potential importance of such a method, it was desirable to further test other model systems. In particular, it was of interest to explore the possibilities of utilizing the charge-shift principle for detecting detergent-binding proteins in complex mixtures.

Materials and Methods

Sodium deoxycholate (Merck) and *N*-cetyl-*N*,*N*,*N*,-trimethylammonium-bromide (Merck) were used without further purification. Triton X-100 (scintillation grade) was purchased from B.D.H., London.

Trypsin (crystalline, 42000 Anson units/g), α-chymotrypsin (crystalline, 850 NF/g) and aprotinin (10 000 K.I.E./ml) were supplied by Novo A/S, Copenhagen. Pronase (B grade) was obtained from Calbiochem.

Proteins. Washed human erythrocytes from fresh or outdated blood were lysed in 10 mM Tris, 1 mM EDTA, pH 8.0, and washed at 4°C in this buffer until free of hemoglobin (300 000 × *g* per min in a Sorvall RC2B-centrifuge, rotor SS 34). Triton extracts of packed membranes (protein concentration 4—5 mg/ml) were prepared as in ref 7. Plasma drawn in 25 mM EDTA (final concentration) was obtained from healthy donors and stored at —90°C. Hemolysate from human erythrocytes was obtained by cell lysis in 10 volumes of 5 mM phosphate pH 8.0, and subsequent centrifugation (Sorvall centrifuge, 15 min at 19 000 rev./min, rotor SS 34).

Antibodies. Rabbit antibodies against human serum proteins (code 100 SF), against human erythrocyte membrane proteins, and against the following human plasma proteins were obtained from Dakopatts A/S, Copenhagen: Prealbumin, albumin, orosomucoid, α-1-antitrypsin, α-1-antichymotrypsin, ceruloplasmin, haptoglobin, α-2-macroglobulin, C3, C4, transferrin, fibrinogen, IgG, IgA, IgM. Rabbit antisera against serum α- and β-lipoproteins were purchased from Behring (Marburg).

Proteolytic degradation. White, human erythrocyte membranes and Triton extracts thereof were made 0.10 M/0.038 M in glycine/Tris, pH 8.7. A combination of trypsin plus α-chymotrypsin, or pronase was added at final enzyme concentrations of 0.2 or 0.1 mg/ml to unsolubilized membranes or Triton extracts, respectively. Incubation was carried out at room temperature (22°C) for 20—24 h. Subsequently, an excess of aprotinin was added to unsolubilized, trypsin/α-chymotrypsin-treated membranes. Membranes were then solubilized in Triton X-100 and the extracts examined.

Charge-shift crossed immunoelectrophoresis. The standard buffer system was 0.1 M glycine, 0.038 M Tris, pH 8.7, in all experiments. 1% (w/v) agarose (type HSA, Litex, Glostrup, Denmark) was used in all cases. Electrophoreses were performed in water cooled (15°C) immunoelectrophoresis apparatuses from Holm-Nielsen Analyseudstyr, Søborg, Denmark. The following agarose-buffer systems were used: (A) 0.5% (v/v) Triton X-100 added to agarose, no detergent in the buffers necessary, (B) 0.5% (v/v) Triton X-100 plus 0.2% (w/v) deoxycholate present in agarose and buffers, (C) 0.5% Triton X-100 plus

0.0125% (w/v) cetyltrimethylammoniumbromide in buffer and gels. Initially, proteins were equilibrated with a surplus of the detergents overnight as described in the original procedure of Helenius and Simons [1]. However, it was found that all observed charge-shifts exhibited by amphiphilic proteins occurred to a comparable extent when the proteins were applied in Triton X-100 alone (no charged detergents), and without pre-equilibration. Moreover, the antigenicities of several membrane and serum proteins were extremely sensitive toward treatment with cetyltrimethylammoniumbromide. The concentration of this detergent in the buffer and gels therefore had to be considerably lowered (0.0125% compared to 0.05% given in the original procedure [1]). After application of the Triton-treated samples, first dimension electrophoresis was performed in 0.15 mm thick agarose gels at 10 V/cm for 60—90 min. 2 μl of crude hemolysate were applied in wells on each side of the agarose plates to monitor the electrophoreses. Native hemoglobin migrates identically in all three electrophoresis systems and thus constitutes a convenient marker *. Electrophoresis was terminated after hemoglobin had migrated 2.0 cm equally in all plates. 1 cm wide agarose strips were then transferred to glass plates (10 × 10 cm) and 0.12 mm thick antibody-containing agarose with 0.5% Triton X-100 (no charged detergents) cast for second dimension immunoelectrophoreses [8]. Immunoelectrophoresis was performed for 16 h at 2 V/cm (glycine/Tris standard buffer, no detergent). Immunoprecipitates were stained for protein with Coomassie Brilliant Blue R [8] and for cholinesterase activity with 1-napthylacetate and Fast Red TR [9].

Two-dimensional charge-shift electrophoresis. First dimension electrophoresis of proteins was performed at 8—10 V/cm in agarose containing 0.5% Triton X-100. Hemoglobin was allowed to migrate 2—3 cm into the gel. Subsequently, 0.5—1 cm wide agarose strips were transferred to new glass plates (7 × 10 cm or 10 × 10 cm) and 1.2 mm thick agarose gels with Triton X-100 plus or minus deoxycholate or cetyltrimethylammoniumbromide were cast on the free parts of the plates (no antibodies). Second dimension electrophoresis was then performed at 4—5 V/cm using the corresponding buffers. Hemoglobin was run 2—3 cm as a marker. The agarose plates were then removed, the proteins fixed in 1.5% (w/v) picric acid dissolved in 20% acetic acid for 20 min, and the plates stained [8].

In the second dimension Triton-deoxycholate system, the absence of deoxycholate in the first dimension gel caused appearance of a cloudy zone proportional to the width of the gel strip (arrows, Fig. 3B). Resolutions were not disturbed provided the strip width did not exceed 1 cm.

Results

Charge-shift crossed immunoelectrophoresis

Plasma proteins. Fig. 1 shows the crossed immunoprecipitation patterns of

* However, we have observed that prolonged incubation (over 2 h at room temperature) of hemoglobin with Triton X-100 results in an alteration of the molecule, possibly through molecular dissociation and denaturation, and the altered protein exhibits marked bidirectional charge-shifts. There is also a concomitant loss of antigenicity (unpublished observations).

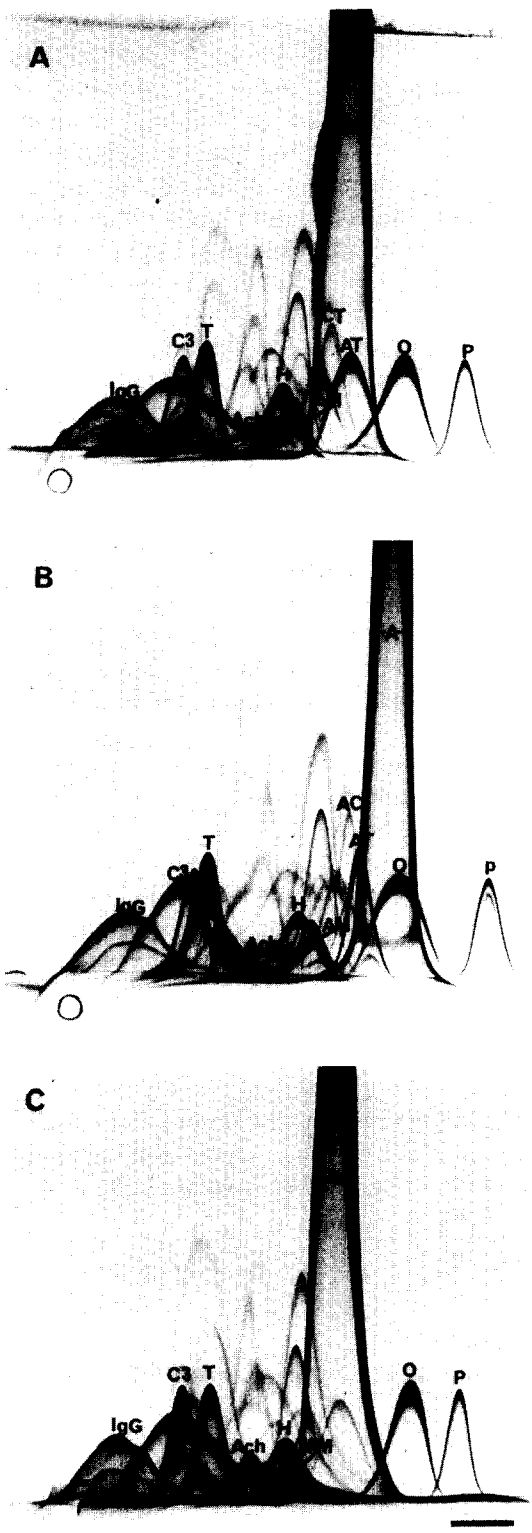

Fig. 1. See opposite page for legend.

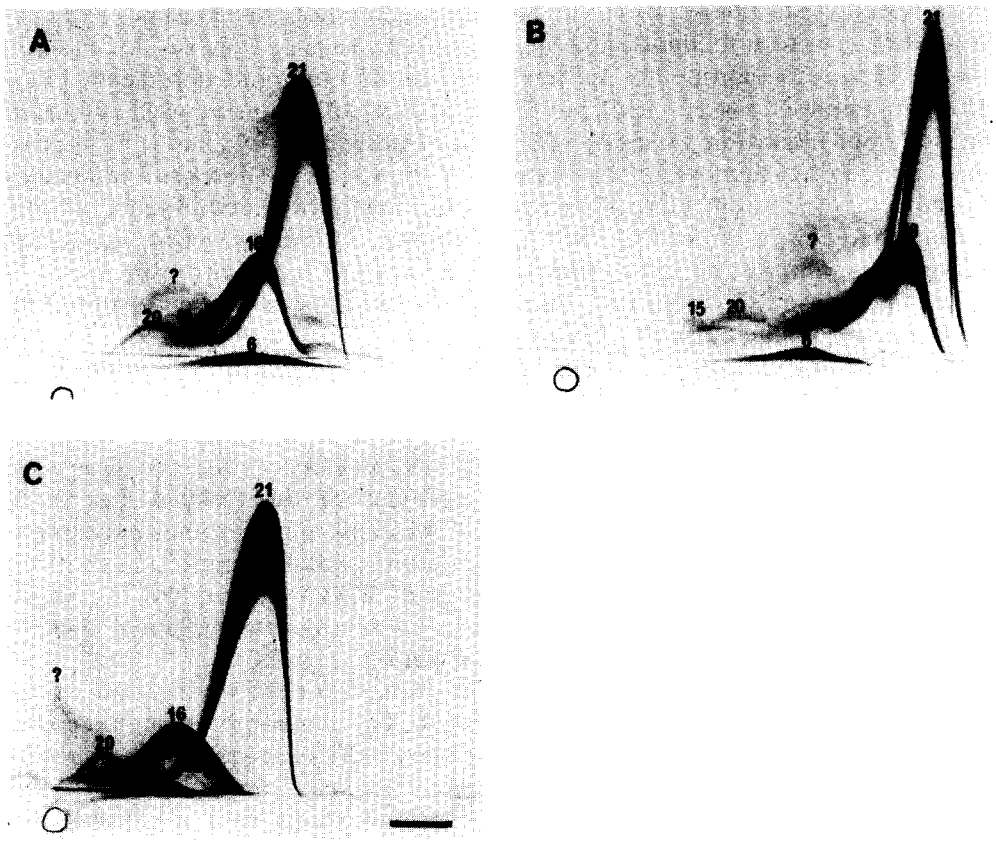

Fig. 2. Charge-shift crossed immunoelectrophoresis of human erythrocyte membrane proteins. 15 μl of unfractionated Triton extract (60 μg protein) were electrophoresed in the first dimension as in Fig. 1. Second dimension gels contained Triton and 10 μl/cm^2 of membrane-specific antibodies. Precipitates are numbered as in ref. 13: No. 6 represents the spectrins, No. 16 the major "intrinsic" membrane protein, No. 21 the major M,N glycoprotein (whose mobility in Triton is somewhat faster than in Berol EMU-043), No. 20 membrane acetylcholinesterase, No. 15 residual hemoglobin. "?" denotes one as yet unidentified amphiphilic membrane protein. Staining as in Fig. 1. The bar represents 1 cm.

plasma proteins electrophoresed equally in the first dimension in gels containing Triton (A), Triton plus deoxycholate (B), and Triton plus cetyltrimethylammoniumbromide (C). No gross denaturing occurs through the charged detergents. Proteins identifiable through comparison with the known reference patterns [10] are indicated on the figures. Further analyses were performed

Fig. 1. Charge-shift crossed immunoelectrophoresis of 2 μl of human plasma proteins. First dimension electrophoresis was performed in (A) Triton, (B) Triton plus deoxycholate and (C) Triton plus cetyltrimethylammoniumbromide. Hemoglobin migration was 20 mm. Second dimension electrophoresis was performed in gels containing Triton and 10 μl/cm^2 of polyspecific antibodies to human serum proteins. The following proteins were identified by comparison with the known precipitation pattern (see ref. 10): prealbumin (P), albumin (A), orosomucoid (O), α-1-antitrypsin (AT), α-1-antichymotrypsin (AC), α-2-macroglobulin (AM), haptoglobin (H), acetylcholinesterase (Ach), transferrin (T), C3 complement component (C3), IgG. α-1-antitrypsin and α-1-antichymotrypsin were not identifiable on plate C. The plates were stained for esterase (1-naphthylacetate, Fast Red TR) and proteins (Coomassie Brilliant Blue). The bar represents 1 cm.

using specific antibodies listed in Materials and Methods. Plasma α- and β-lipoproteins were not identifiable in this system because they do not precipitate with specific antibodies in the presence of non-ionic detergents [11]. Albumin exhibits a very small bidirectional shift of 5 mm in each direction (hemoglobin migration 20 mm), which can readily be explained by the presence of a limited number of high affinity binding sites for detergent molecules present on this protein [12]. No single identifiable protein exhibited a bidirectional charge-shift exceeding that of albumin. Use of specific antibodies disclosed an interesting phenomenon in the case of α-1-antitrypsin and α-1-antichymotrypsin. Both shifted markedly in the presence of cetyltrimethylammoniumbromide, but not in deoxycholate.

Erythrocyte membrane proteins. Fig. 2 depicts the crossed immunoelectrophoresis patterns of human erythrocyte membrane proteins present in an unfractionated Triton extract analysed in the same system. The major precipitates of the normal patterns (A) have been identified previously [13]. No disappearance of precipitates is observed in the presence of the charged detergents (Figs. 2B and 2C). However, the "intrinsic" proteins appear somewhat altered in the presence of deoxycholate, the immunoprecipitates exhibiting splitting of limbs and peak-doubling. Spectrin in a crude EDTA extract and the major M,N glycoprotein prepared as in refs. 7 and 14 exhibited comparable electrophoretic behaviours as in the unfractionated Triton extracts.

Table I summarizes the charge-shift values found for the erythrocyte membrane proteins and plasma proteins analysed. The membrane proteins corresponding to precipitates 16, 20 and 21 clearly shift bidirectionally, in accordance with their known classification as "intrinsic" membrane proteins [13,15–17]. Spectrin also exhibits a bidirectional charge-shift which is, however, less pronounced than that observed for the "intrinsic" proteins.

Two-dimensional charge-shift electrophoresis

Fig. 3A depicts a two-dimensional electrophoresis of plasma proteins in Triton alone. When second dimension electrophoresis is performed in Triton

TABLE I

INCREASES AND DECREASES IN ELECTROPHORETIC MIGRATION (in mm) OF HUMAN ERYTHROCYTE MEMBRANE PROTEINS AND HUMAN PLASMA PROTEINS

Hemoglobin migration 20 mm in all cases.

Protein	Anodic shift (mm)	Cathodic shift (mm)
Major "intrinsic" membrane protein	22	13
Major M, N glycoprotein	20	6
Membrane acetylcholinesterase	12	8
α-Lipoprotein (as determined by two-dimensional charge-shift electrophoresis)	20	12
β-Lipoprotein (as determined by two-dimensional charge-shift electrophoresis)	15	6
Spectrin	7	15
Albumin	5	5
α-1-Antitrypsin	0	25
α-1-Antichymotrypsin	0	25
Other plasma proteins	$\leq$ 5	$\leq$ 5

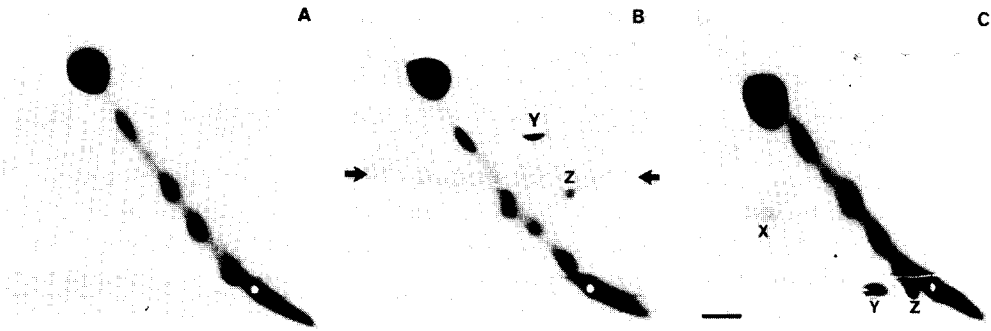

Fig. 3. Two-dimensional charge-shift electrophoresis of human plasma proteins. First dimension electrophoresis (anode left) was performed in Triton. Second dimension electrophoresis (anode at top) was performed in (A) Triton, (B) Triton plus deoxycholate, (C) Triton plus cetyltrimethylammoniumbromide. X, Y and Z denote proteins exhibiting significant changes in migration in the presence of the charged detergents. Hemoglobin migration was 20 mm in both dimensions. Fixation and staining: Picric acid and Coomassie Brilliant Blue, respectively. The bar represents 1 cm.

plus deoxycholate (Fig. 3B), two proteins migrate out of the standard diagonal. In Triton plus cetyltrimethylammoniumbromide (Fig. 3C), three protein spots X, Y, and Z are seen below the diagonal, representing cathodally shifting entities. Two (Y and Z) correspond to the anodally shifting proteins.

The proteins exhibiting charge-shifts were isolated by placing unstained plates above stained plates which had been run in parallel, and sectioning at the corresponding positions. Subsequent immunochemical analyses in detergent-free agarose permitted identification of the proteins. X represents plasma α-1-antitrypsin and α-1-antichymotrypsin, and Y and Z were identified as apoproteins of α- and β-lipoprotein, respectively.

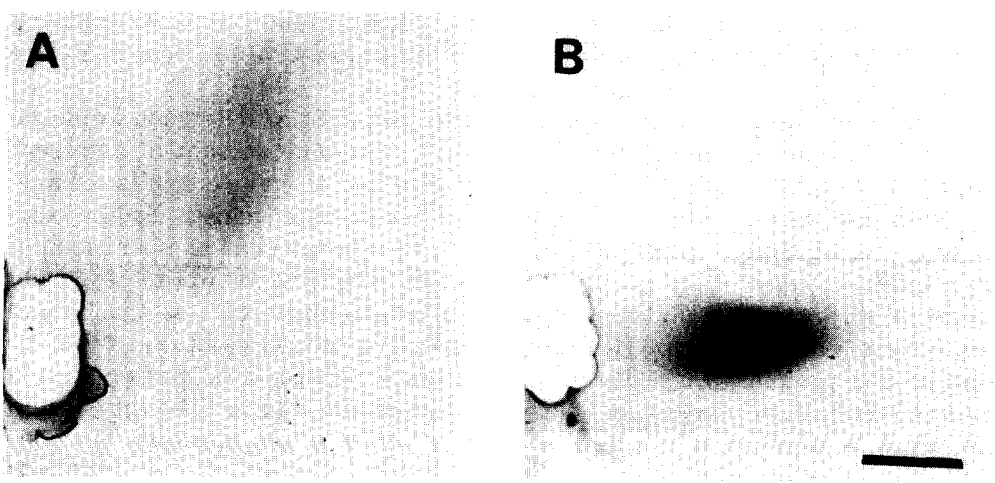

Fig. 4. Detection of residual, amphiphilic peptide(s) derived from extensively proteolysed human erythrocyte membrane proteins. 130 μl of a Triton extract of erythrocyte membranes were treated with pronase and then analysed two-dimensionally. First dimension electrophoresis (anode to the right) was performed in Triton, second dimension electrophoresis (anode at top) in Triton (A) and in Triton plus cetyltrimethylammoniumbromide (B). Hemoglobin migration was 30 mm in the first dimension and 25 mm in the second dimension. Fixation and staining as in Fig. 3. The bar represents 1 cm.

The bidirectional charge-shift found for the apoproteins are in accordance with their known detergent-binding character [18,19].

Erythrocyte membrane proteins were not well resolved in two-dimensional charge-shift electrophoresis because the electrophoretic migration of the major protein entities overlap.

Amphiphilic peptides obtained by proteolytic cleavage of membrane proteins. Apolar regions of "intrinsic" membrane proteins embedded within the lipid matrix will resist cleavage by proteolytic enzymes when isolated membranes are extensively degraded. Moreover, Triton solubilization of membranes should result in replacement of lipids by the detergent molecules, which may be expected to at least partially protect the relevant peptide regions from total degradation [20]. The possible utility of charge-shift electrophoresis for detecting such peptides was explored using extensively degraded erythrocyte membrane proteins. Indeed, two-dimensional charge-shift electrophoresis reveals that fixable and stainable material of β-mobility is still present in such membrane proteolysates (Fig. 4A). Second dimension electrophoresis in Triton plus cetyltrimethylammoniumbromide causes all material to shifts cathodically yielding a single peptide spot (Fig. 4B). A marked anodal shift was also found in the Triton-deoxycholate system. This was observed in all tested systems, i.e. proteolysis of intact and solubilized erythrocyte membranes, with both α-chymotrypsin plus trypsin or pronase. The amphiphilic peptide(s) did not precipitate with polyspecific antimembrane antibodies in crossed immunoelectrophoresis.

Discussion

The presented results supplement the data of Helenius and Simons [1]. In particular, the lack of significant, bidirectional charge-shifts of all plasma proteins detectable in our systems other than the lipoproteins constitute evidence that bidirectional charge-shifts of hydrophilic proteins would represent rare exception.

Proteins of similar size binding equivalent amounts of detergent would be expected to exhibit comparable charge-shifts. The same amount of detergent bound to a much larger protein would be expected to induce smaller charge-shifts because of the reduction in density of the surplus charges. On the basis of our present study, it may at this stage prove convenient to operationally distinguish between three groups of proteins. The first comprises typically hydrophilic protein exhibiting bidirectional charge-shifts of less than 5 mm (e.g. serum proteins except the lipoproteins). The second comprises typically amphiphilic proteins whose anodic and cathodic changes in migration exceed 12 and 6 mm, respectively, when hemoglobin migrates 30 mm. The third group comprises borderline cases such as spectrin, where it will be necessary to quantitatively determine the extent of Triton binding using other methods. With respect to spectrin, it is noteworthy that previous studies have indeed disclosed that the protein possesses discrete detergent-binding areas [6,21].

As mentioned by Helenius and Simons [1], it should be stressed that a protein must shift bidirectionally in order to be classified as specifically Triton binding. Unidirectional shifts, as exhibited by α-1-antitrypsin and antichymo-

trypsin, indicate specific binding of one of the charged detergents to the proteins, probably through electrostatic interactions.

A major aim of this work was to provide methods for detecting detergent-binding proteins in complex mixtures without their prior purification. This situation may arise in a variety of membrane studies. When a poorly characterized membrane system is being investigated, two-dimensional charge-shift electrophoresis may initially yield information on the minimum number and electrophoretic mobilities of major, detergent-binding proteins. The concentration of cetyltrimethylammoniumbromide may be increased in such studies if accentuation of cathodal shifts is desired. When polyspecific antisera to the protein mixtures are available, charge-shift crossed immunoelectrophoresis should rapidly reveal the presence of amphiphilic moieties. In particular, the procedure should prove useful in the case of minor membrane proteins, whose preparative isolation may pose problems. Membrane enzymes fall under this category, and the analysis of erythrocyte membrane acetylcholinesterase illutrates the feasibility of the experimental approach.

The origin and molecular nature of the detergent-binding peptide(s) derived from proteolysed human erythrocyte membrane proteins is currently being investigated. It is apparent that the precise peptide composition of a seemingly homogeneous spot observed in one or two-dimensional electrophoresis still must be established using other methods, as co-migration of different peptides in the agarose systems is readily conceivable. In the given case, two-dimensional charge-shift electrophoresis is preferable to the unidirectional system for demonstrating amphiphilicity of the degradation product(s) for two reasons. Firstly, far greater volumes of the samples can be applied. Secondly, the net mobility of the peptide(s) in Triton plus cetyltrimethylammoniumbromide is zero (Fig. 4B): the material therefore remains in the application well in one-dimensional electrophoresis and is difficult to detect.

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