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Review

Electromigration for separations of protein complexes

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Abstract

This paper describes electromigration of complexes, consisting of two or more proteins and non-covalently associated peptides. Relatively small complexes (M_r<1 000 000) can be resolved in sieving matrices. Large complexes are separated in free liquid systems. Examples of separation are given using native gels, denaturing gels and special formats thereof: blue native PAGE and gels incorporating a transversal temperature gradient. Both preparative and analytical applications are discussed as well as separations leading to mechanistic models of protein interaction. Carrier-free electrophoresis is represented by capillary zone electrophoresis, free-flow electrophoresis and density gradient electrophoresis. Emphasis is put on the free liquid separation of clathrin-coated vesicles and proteasomes.

Keywords: Reviews; Density graident electrophoresis; Proteins

Contents

Introduction

The study of electromigrating native protein complexes is a relatively unexplored area. In spite of its evident importance, a recent review [1] mentions future developments only and elsewhere [2] it is stated that capillary zone electrophoresis will have applications for the separation of protein complexes at neutral pH. A highly recommendable methodology book [3] mentions the separation of native proteins just briefly.

Protein complexes, in particular those formed by

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non-covalent interactions, generally require electrophoretic native conditions at pH values that are compatible with the stability of the association. As for molecular sieving, matrices like non-denaturing polyacrylamide gels, are limited to about molecular mass (M_r) 1 000 000 when pore size becomes the impediment. To overcome this obstacle, free liquid electromigration is the inevitable next step for the separation of large supramolecular structures. In cases where electric charge is not sufficient for discrimination, one might consider adding sieving agents to enhance separation. Theoretical considerations by Chrambach and Radko [4] predict that resolution in polymer networks is insufficient for the particle size range of 20–1200 nm and he judges that preparative size separations are as yet not feasible with presently available apparatuses due to the polymers impact on the (high) viscosity of the free liquid medium [5]. Capillary zone electrophoresis (CZE) can be considered an ideal format for complex separation. Corradini reviews at length the separation of single proteins but mentions only the separation of albumin dimers [6]. A recent paper symposium [7] on carrier-free electrophoresis gave little information, indicating once more that electrophoretic separation of protein complexes is still in its infancy.

In this review we present examples of electromigrating protein complexes, consisting of two proteins (peptides) and more, partly as well as on ongoing investigations in the authors' institute and partially overseeing results obtained by others. A division is further made between gel electrophoresis and free liquid electrophoresis. We will refrain from dealing with protein aggregates obtained through chemical cross-linking for which a relevant review is due to Loster and Josic [8].

2. Size sieving of protein complexes in gelled matrices

2.1. Separation of complexes in native gels

In the absence of sodium dodecyl sulfate (SDS), it appeared possible to electrofocus, using standard ampholines on a 0.8% agarose gel, the tetanus toxoid/anti-tetanus toxoid – as well as the transferrin/anti-transferrin antibody (Ab) complex from their constituent proteins. The larger complexes $(antigen)_nAb$ did not penetrate into the gel however [9]. The separation of a heterotrimeric complex of periplasmic chaperone Fae E (itself a homodimer, isoelectric point, p*I* 9.7) and Fae H (p*I* 4.2) was separated by native polyacrylamide gel electrophoresis (PAGE) (8 % at pH 9.3) [10]. Also from *E. coli*, a complex consisting of Fae H and Fae I (2:1) was separated.

Proteasome is the subject of active investigations (see Section 3.2). Briefly, the proteasome, a multicatalytic endopeptidase, is of about M_r 740 000. It incorporates the major protein degrading machinery apart from the endosomal/lysosomal compartments. The proteasome [11] contains four stacked rings, the top and bottom rings consisting of non-catalytic α-subunits. In between are two rings made up of catalytic active β-subunits. Proteolysis takes place within the interior aqueous cavity. Both α and β subunits are subdivided in 14 different subunits. There exist moreover three other non-essential, but inducible (by gamma-interferon) subunits: low-molecular-mass polypeptide (LMP) 2, LMP 7 and multicatalytic endopeptidase complex-like-1 (MECL 1) that can replace some of the β subunits of the proteasome. It was surmised that this would lead to the formation of subpopulations of proteasomes [12]. Proteasomes, first purified by conventional (immunoaffinity) chromatography, were separated on a non-denaturing 4.5 % PA gel in Tris–borate buffer at pH 8.3 [13]. Two closely spaced slowly migrating bands (corresponding with 26 S particles) and two other, equally closely spaced, somewhat more rapidly migrating bands were detected by immunoblotting (Fig. 1). These studies followed earlier reports by Hoffman et al. [14] closely. The latter authors demonstrated the existence of a 26 S_{slow} and 26 S_{fast} form (that hydrolyse ubiquitin-conjugated proteins) and a 20 S_{slow} and 20 S_{fast} population, respectively, after overlaying the native gels with fluorogenic peptides.

Schmidtke et al. [15] were furthermore able, while using native 4–15 % PA gels, to separate precursors of proteasome assembly, i.e., the 13 S and 16 S particles.

When recombinant DNA is overexpressed in bacteria the resultant protein is often found in an

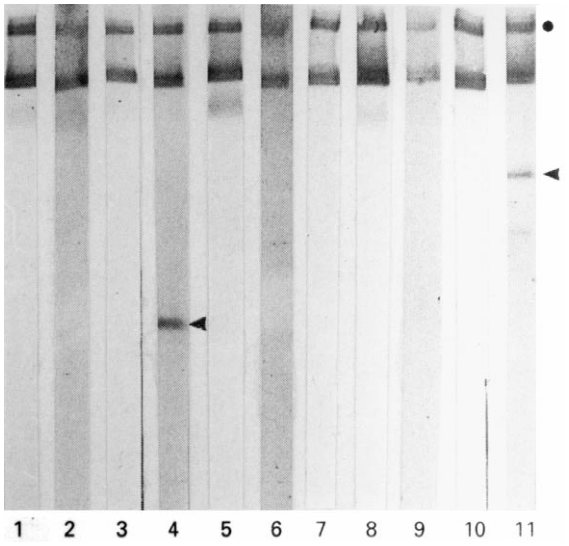


Fig. 1. Proteasome subunits in cell extracts. Supernatants from HeLa extracts were subjected to non-denaturing PAGE and after blotting on to nitrocellulose inclubated with Mabs as described in Ref. [13]. The uppermost doublet of bands (26 S proteinase) is marked by ●. The unmarked doublet is proteasomes. Reproduced from Ref. [13] with kind permission of the publisher.

aggregated form in inclusion bodies. These proteins require refolding for activity. The nativity of a refolded structural gp 41 protein (from the HIV-1 envelope) was assessed by a band-shift assay using native gel electrophoresis. Fab fragments, recognizing (conformational) epitopes on gp 41 were allowed to bind to refolded gp 41. When binding took place, extreme band shifts occurred [16].

Recombinant α_1-antitrypsin (α_1-AT), produced as inclusion bodies, gives rise to monomers, dimers and higher oligomers after refolding as native PAGE revealed. It was assumed that through so-called ''loop-sheet polymerization'' the loop of one molecule of α_1-AT is inserted into the A-sheet of a second α_1-AT molecule. The ensemble remained intact during electrophoresis [17].

Native PAGE can also be used to follow conformational alterations, as is illustrated in Figs. 2–5. Using native PAGE in the presence of 0.1 % NP-40 at pH 8.0, the thermal ''melting'' of complexes consisting of major histocompatibility (MHC) heavy chain, β_2-microglobulin and peptides, was monitored [18]. Empty major histocompatibility complex molecules class I molecules present on the surface of RMA-S (cultured at 26°C) cells were first loaded with the iodinated peptides APGNYPAL, FAPGNYPAL (SEV-9) and RGYVYQGL (VSV-8), respectively. The thermostability of these peptide-loaded MHC class I molecules was assessed using temperature gradient native polyacrylamide gel electrophoresis. A linear temperature gradient perpendicular to the direction of electrophoresis yielded a graphical representation of the melting of MHC class I molecules. The class I signal disappeared when the peptide melted out of the binding groove, and gave rise to a second signal due to released peptide. Figs. 2–5 show the melting of these peptides. APGNYPAL-loaded class I molecules melted at 11°C with considerable release even at 0°C. VSV-8-loaded class I molecules melted first at 36°C, whereas SEV-9-loaded molecules melted at about 22°C. A discrimination between the binding of SEV-9 to K^b and D^b molecules was seen in the melting patterns. Always on top, barely entering the gels, aggregated MHC class I molecules were detected, but these aggregates were temperature labile as well.

2.2. Agarose gels can be used for separation of large protein complexes

Going to ever increasing complexity, clathrin-coated vesicles (CCVs) are involved in the intracellular trafficking of receptors and enzymes. CCVs contain at least adaptor–receptors, adaptor proteins plus the triskelions of clathrin [19]. Non-sieving agarose gels of very low concentration (0.15 %) have been used for the purification of these 80 nm diameter particles [20,21]. The presence of cargo proteins in these CCVs have been assessed, in particular lysosomal enzyme precursors [22,23].

2.3. Separation of oxidative phosphorylation complexes by blue native PAGE (BNPAGE)

Very elegant by its simplicity is a method developed by Schagger and co-workers [24–26]. From a mitochondrial preparation, or in the case of muscle directly from the total pelletable fraction all respiratory chain complexes can be isolated. These complexes are described individually in [25] often in connection with blue native PAGE. For mammals, the constitution of these complexes are: (i) complex

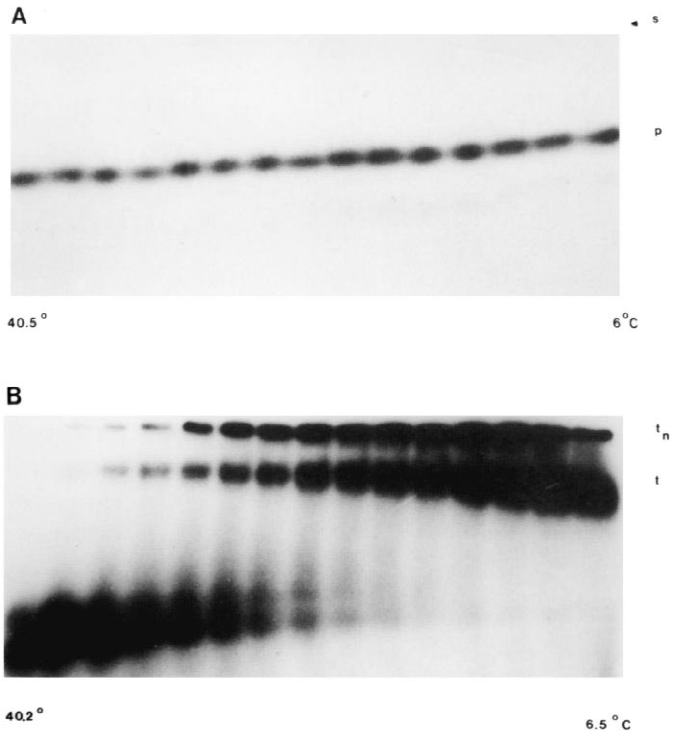


Fig. 2. TG native PAGE of murine major histocompatability complex (MHC) class I molecules loaded with the nonamer Sendai virus (SEV)-9. (A) Electrophoretic mobility of ^{125}I-labeled SEV-9 (sequence FAPGNYPAL). Radiolabel was detected by autoradiography. (B) Thermal melting of MHC class I molecules. RMA-S cells were cultured for 48 h at 26°C and ''empty'' class I molecules loaded with the nonamer peptide ^{125}I-labeled SEV-9. The post-nuclear supernatant was subjected to TG native PAGE. The TG ranged from 6.5 to 40.2°C. t_n=Aggregated MHC class I; t: MHC class I; p: peptide released from t; p_n: peptide released from t_n. Reproduced from Ref. [18] by kind permission of the editor.

I, NADH: ubiquinone oxidoreductase, 40 different subunits, (ii) complex II, succinate–ubiquinone reductase, four different subunits, (iii) complex III, ubiquinol–cytochrome *c* oxidoreductase, 11 subunits, (iv) complex IV, cytochrome *c* oxidase, 13 subunits, (v) complex V, F_1F_0-ATPase, 16 subunits.

Separation is performed at 4–7°C and at a pH of 7.5 since several complexes are unstable at the standard pH (9.0) of the Laemmli SDS–PAGE method [27]. Fig. 6 shows an example of complex separations. The elegant method deserves an extended description. Neutral detergents are used to solubilize the mitochondrial proteins and salts (K^+, NH_4^+, divalent cations) are substituted for 6-aminocaproic acid (at 0.7 *M* for a high dielectric constant) since otherwise Coomassie stain starts to precipitate [25]. Due to its pK_1=10.7 and pK_2=4.4, caproic acid is almost completely neutral and does not migrate at pH 7.5 [24]. Coomassie dye fulfils four functions: (1) it binds to the surface of proteins by apolar interactions making the proteins of negative charge, resulting in anodic migration. (2) Proteins covered by Coomassie repel each other and have reduced aggregation properties. (3) Although neutral detergents are used in the initial preparative steps, they are absent in the gel. In the absence of detergent, Coomassie binds strongly to the proteins since it is of hydrophobic nature and barely soluble in water. (4) The stained complexes remain visible during PAGE.

Six to 13 % PA gels are used and the larger complexes soon come to a standstill due to limiting pore size.

In a later development, native PAGE but named colorless native PAGE (CNPAGE) was combined with BNPAGE [26]. Whereas BNPAGE causes a

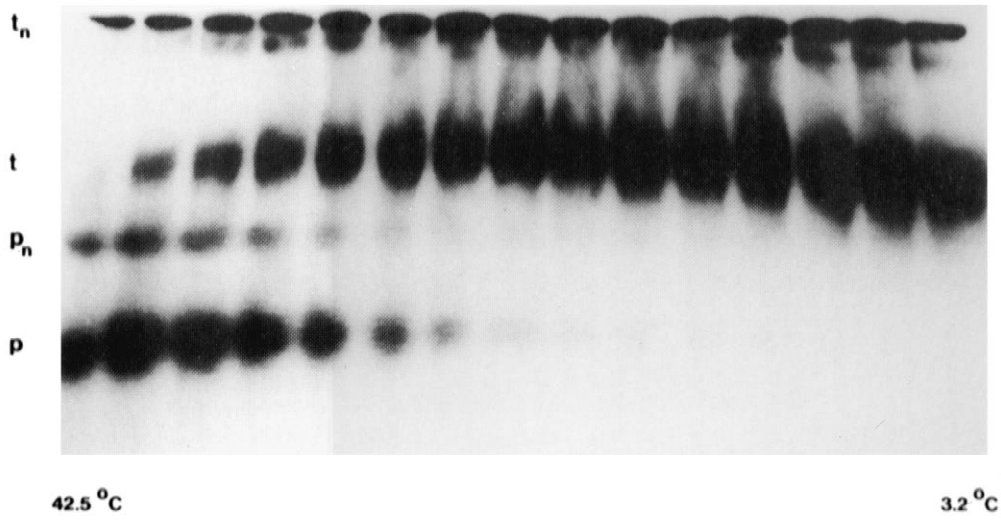


Fig. 3. TG (1 h) native PAGE of MHC K^b and D^b molecules loaded with SEV-9. Experimental conditions as in Fig. 2. The TG ranged from 3.2 to 42°C. Reproduced by kind permission of the editor [18].

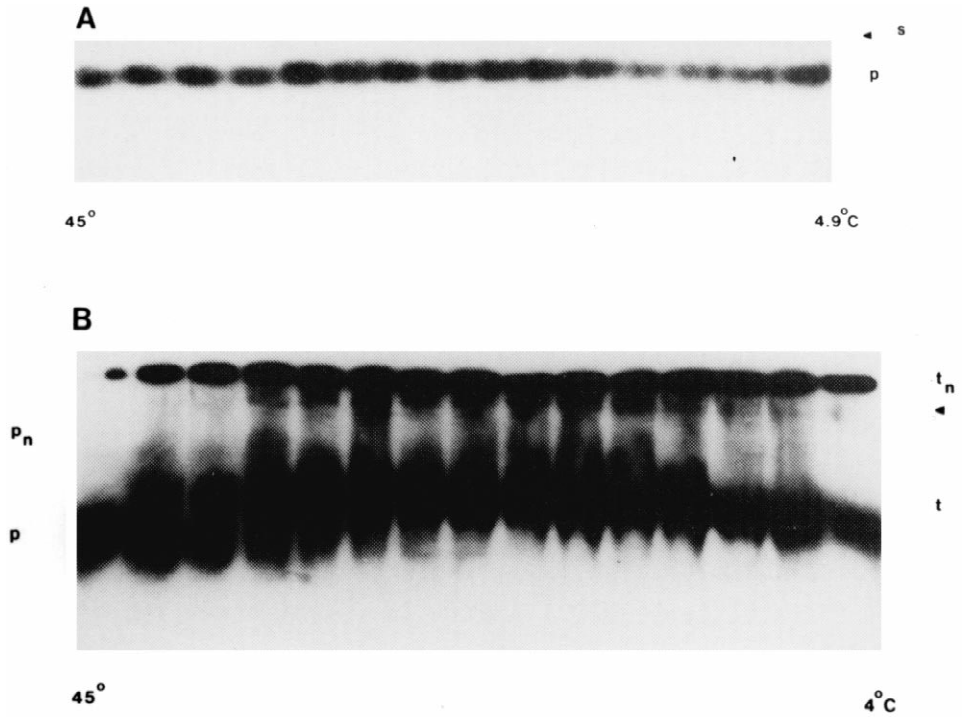


Fig. 4. TG native PAGE of MHC K^b molecules loaded with the octamer ^{125}I-labeled vesicular stomatitis virus (VSV)-8. (A) Electrophoretic mobility of the peptide VSV-8 in TG native PAGE with the TG ranging from 4.9 to 45°C. p: Peptide. (B) TG native PAGE of MHC K^b molecules loaded with VSV-8. Experimental conditions as in Fig. 2, except that the octamer VSV-8 was loaded onto ''empty'' class I molecules. The TG ranged from 4 to 45°C. Arrow: minor species; t_n: aggregated MHC class I; t: MHC class I; p: peptide released from t; p_n: peptide released from t_n. Reproduced by permission of the editor [18].

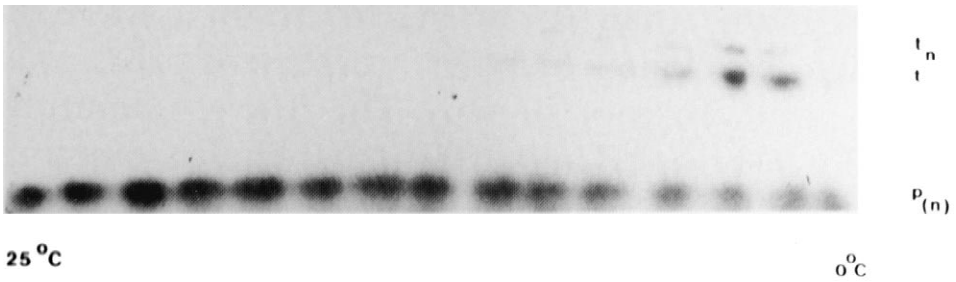


Fig. 5. Extreme thermolability of APGNYPAL-loaded MHC class I molecules. The TG ranged from 0 to 25°C. Experimental conditions as in Fig. 2. Reproduced by kind permission of the editor [18].

charge shift through binding of negatively charged Coomassie dye to the protein complex, CNPAGE exploits the intrinsic charge of the complex. This allowed separation according to two independent parameters when two-dimensional CNPAGE (first dimension) followed by BNPAGE was employed.

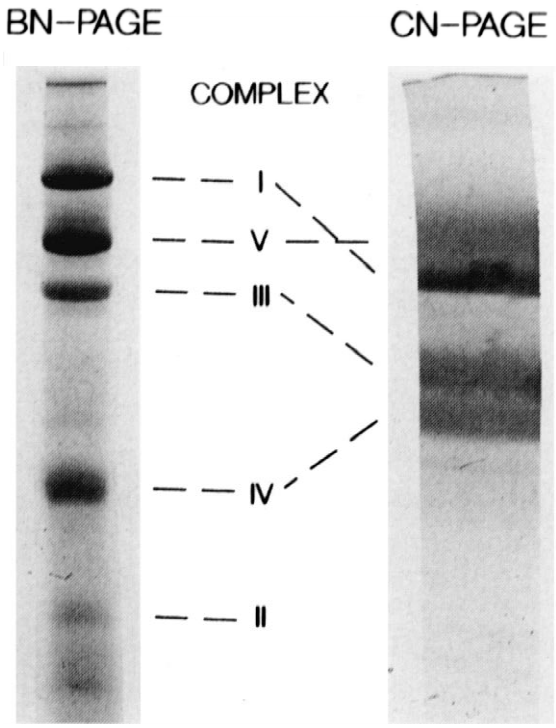


Fig. 6. Comparison of resolution of first-dimension blue native PAGE and colorless native PAGE using solubilized bovine heart mitochondria. The most prominent bands were the membrane protein complexes I–V of oxidative phosphorylation. Reproduced from Ref. [26] with permission.

CNPAGE has a lower quality of separation but in combination with BNPAGE beautiful separations of the mitochondrial complexes were obtained. After electrophoresis and cutting out of bands and spots, the catalytic activity of the mitochondrial complexes generally remained intact.

Typical applications have been for oxphos complexes in Parkinson's disease [28], mitochondrial encephalomyopathy [29], deficiencies of complex V in Alzheimer's disease [30] and oxphos complexes from cell lines [31]. Other BNPAGE applications report on plant mitochondrial protein complexes [32], multiprotein (glycoprotein IIb–IIIa complex, M_r 330 000) a receptor for fibrinogen and the von Willebrand factor from platelets [31].

2.4. Separation of complexes in denaturing gels

2.4.1. Protein complexes unusually stable in SDS

Asundi and Carey [33] used SDS-PAGE to show that *N*-syndecan, thought to play an important role in morphogenesis, is capable of self-association and forms non-covalent dimers and sometimes tetramers, unusually stable in SDS (2%)-PAGE.

Tulp and Verwoerd [34] exploited temperature gradient PAGE (TGPAGE), where a transversal linear temperature gradient is perpendicular to the electrical field in PA gels (see also Section 2.1). It was applied to the ''melting'' of non-covalently bound dimers, stemming from IgG_1. IgG_1, upon reduction at room temperature by β-mercaptoethanol, gives rise to a non-covalent dimer of the heavy chain (HH), as well as to a heavy–light chain (HL) dimer in SDS-containing buffers. Both dimers could be melted in monomers using a 40–75°C TG

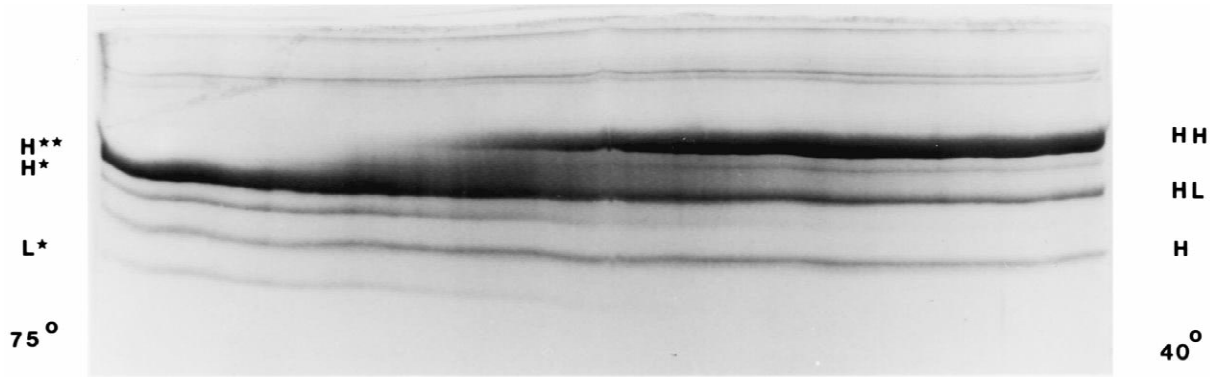


Fig. 7. TG SDS-PAGE of mildly reduced human monoclonal IgG_1. The temperature gradient ranged from 40 to 75°C. HH=Dimer of heavy chain. HL=Dimer of heavy and light chain. For details see Ref. [34]. Reproduced by kind permission of the editor [34].

under conditions of SDS-PAGE, Fig. 7 (for details, see Ref. [34]). It should be noted that only IgG_1 and IgG_2 do so while IgG_3 and IgG_4 are split directly into monomers of H and L at room temperature under reducing conditions.

MHC class II molecules, loaded with ''high affinity'' peptides (of about 12–20 amino acids length) in late endosomal/lysosomal compartments [35], are exceptionally stable in SDS containing (reducing or not reducing) solutions at room temperature. Only class II molecules loaded with so called C-terminal leupeptin induced peptides (CLIPs) or other ''low-affinity'' peptides derived from the chaperone, the invariant chain, decompose rapidly in the α- and β-chain and peptide [36]. Fig. 8 shows the stability of trimeric peptide, α- and β-chains in the non-boiled state as well as its decomposition after boiling. Also shown in Fig. 8 is the stability of a MHC class II construct, where the β-chain was attached to green fluorescent protein [37]. The peptide' s presence of varying composition is not shown in the fluorograph of Fig. 4 due to inefficient labeling.

2.4.2. Separation of protein complexes with intact disulfide bonds

It goes without saying that protein complexes held together by S–S bridges remain intact during non-reducing PAGE. The separation of IgG_3 has found its way in at least two textbooks on electrophoresis, clearly for didactic purposes [3,38]. Both heavy–heavy chain and heavy–light chain have inter-

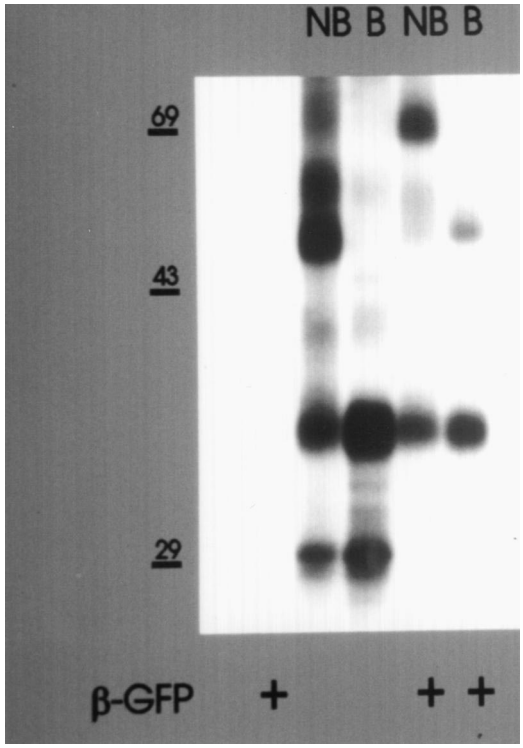


Fig. 8. Biochemical analysis of cell surface MHC class II molecules. MelJuSo cells were transfected with the HLA-DR1 β-GFP construct [37]. After lysis, class II molecules were immunoprecipitated using a Mab as described in Ref. [37]. The isolate was boiled (B) or not boiled (NB) and separated by SDS-PAGE.

molecular bonds. In one and the same SDS-PA gel, slots were filled with aliquots containing β-mercaptoethanol treated IgG_3 next to slots filled with IgG_3 in the absence of reducing agent. Lateral diffusion of mercaptoethanol gave rise to partial reduction of neighbouring non-reduced samples and ''connecting arcs'' were seen after Coomassie staining. From these it could be deduced where intrachain disulfide binding occurs [39]. Of the numerous homodimers, held tightly by S–S bridges, only two will be mentioned. Neefjes et al. [40] subjected HepG2 cells to ^{35}S-methionine pulse-chase regimes. Using SDS-PAGE under non-reducing conditions, these authors established that during early biosynthesis already in the ER dimerization of the transferrin receptor (TfR) occurred. Moreover it could be shown that the acquisition of transferrin binding capacity coincided with covalent TfR-dimerization, Fig. 9 and the legends for its explanation.

Thomas et al. [41] made clear that in drug-resistant B-cell chronic lymphocytic leukemia the baxprotein (p 21 and p 18) formed protein complexes linked by S–S bridges. These complexes, ranging from M_r 36 000 to 42 000, were analysed by two-dimensional gel electrophoresis (first under non-reducing conditions) and were found to be homo- and heterodimers. Following addition of 9-amino-20(s)-camptothecin, apoptosis ensued while the complexes were upregulated.

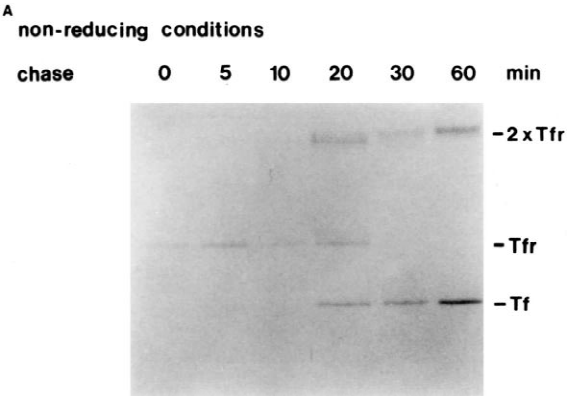


Fig. 9. Interactions of Tf and TfR early during biosynthesis. HepG2 cells were labeled for 5 min with ^{35}S-methionine and chased for the times indicated. TfR was immunoprecipitated and analyzed by SDS-PAGE under non-reducing conditions. 2x TfR= Disulfide-linked homodimer. Tf binding is observed as soon as the covalent TfR is formed. Reproduced from the Journal of Cell Biology, 111 (1990) 1383–1392 by copyright permission of The Rockefeller University Press.

3. Separation of protein complexes by carrier-free electrophoresis

3.1. Capillary electrophoresis (CE)

CZE with its inherent high plate number is as yet not used intensively for the analytical separation of protein complexes, Nielsen et al. [42] separated an antibody–antigen complex: human growth hormone (HGH, M_r 22 000, p*I* 5.2) and a monoclonal antibody (IgG, M_r 150 000 p*I* between 7.2 and 8.0) were separated from the HGH–IgG complex at pH 8.0. Even IgG–$(HGH)_n$ complexes were visible in the electropherogram.

Other examples of Ab–antigen migration can be found in [43]. Shimura and Karger [44] developed an affinity probe capillary electrophoresis method using the principles of isoelectric focusing (IEF). These authors specified that ''an affinity probe must form a kinetically stable complex with respect to the time of the separation and should be electrophoretically resolvable from the complex''. To that end a Fab fragment was labelled with tetramethylrhodamine–iodoacetamide and then allowed to bind to recombinant growth hormone.

In order to study the dissociation constant of deoxyribonuclease I and G-actin, CZE was used for the determination of the K_d in the nanomolar range. DNAse I forms a 1:1 complex with actin and inhibits actin polymerization in vitro [45].

Zhu et al. [46] overcame the similar mass/charge ration of the monomer, dimer and trimers of albumin by sieving according to size in a capillary filled with 5% of the linear polymer polyethylene glycol (PEG). As is the method's nature it remains limited to analytical procedures.

Tsuji [47] used a SDS (non-acrylamide) polymer filled capillary (commercially available) to sieve the monomer, dimer, trimer and tetramer forms of recombinant bovine somatotropin down to baseline resolution under non-reducing conditions. It was

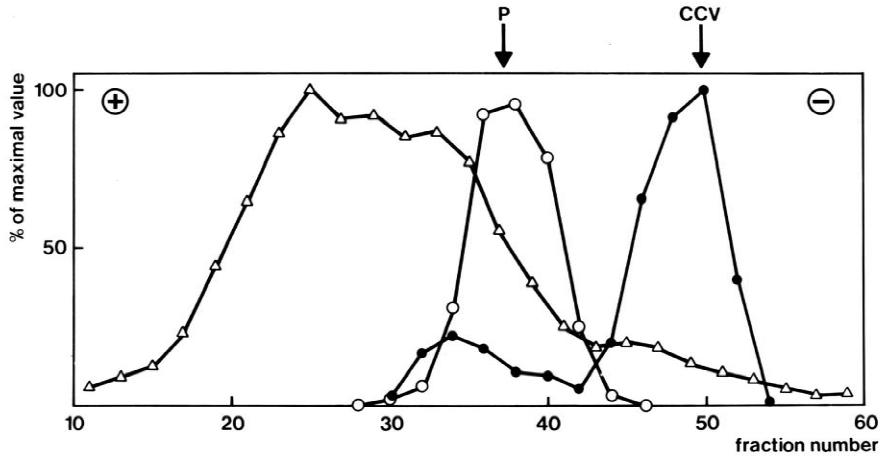
assumed that the dimeric and higher forms were the result of intermolecular disulfide bonding during the refolding process of the recombinant protein prior to electrophoresis. These multimers should be considered artifacts however.

3.2. Free-flow electrophoresis (FFE)

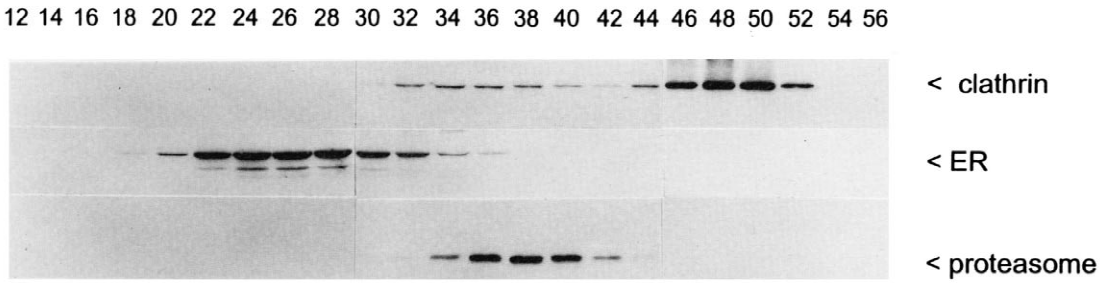
Morris and co-workers [48,49] have separated clathrin-coated vesicles by FFE and were able, after treating the purified CCVs with antibodies to coat-associated proteins, to induce a small electrophoretic shift of a second population of CCVs [48].

3.3. Density gradient electrophoresis (DGE)

DGE [50] has recently been revitalized and found particularly useful applications in subcellular organelle separations [35,51–55]. As an unexpected spin-off, at least two protein complexes became simultaneously separated. Figs. 11a and b show that within 20 min of electrophoresis in a low (195 μS/cm) conductivity buffer both clathrin-coated vesicles as well as proteasomes were purified. Directly from a postnuclear supernatant, derived from HeLa cells, these complexes came free from the bulk of organelle protein. It is evident from Fig. 10a that upon longer electrophoresis, both CCV and protea-



(a)



(b)

Fig. 10. Rapid DGE separation of HeLa cell organelles. For details see Ref. [51]. Proteasomes and clathrin were determined after immunoblotting of the fractions obtained through 20 min DGE. (a) (△–△) protein; (○–○) proteasomes (P); (●–●) clathrin-coated vesicles (CCVs). (b) Immunoblot of organelle fractions obtained by DGE. Numbers denote fraction number. ER (gp 96); proteasome (Mab directed at the α_1-subunit of the proteasome); clathrin was detected using the Mab directed at the heavy chain. Reproduced by kind permission of the editor [51].

somes were to become completely free from the organelles. Clathrin – as the immunoblot of Fig. 10b shows – came into two populations: a minor one of clathrin-coated pits at the plasma membrane (PM) and a major population of CCVs proper. Proteasomes peaked at fraction 38. Preliminary experiments (in collaboration with Dr. P. Kloetzel, Berlin, Germany), starting right from purified proteasomes, showed the feasibility of separating subclasses of proteasomes. These separations were performed in sucrose density gradients containing 3-[*N*-morpholino]propanesulfonic acid (MOPS)/γ-aminobutyric acid (GABA) buffers of low (70 μS/cm) conductivity.

As a second example of DGE, Fig. 11 shows the almost complete separation of CCVs from other vesicles. First lysosomes and mitochondria, derived from the human melanoma cell line Mel JuSo, were removed by flotation of a postnuclear supernatant on a 0.25 *M*/0.86 *M* interphase through density gradient centrifugation. Thus endosomes, Golgi, endoplasmic reticulum (ER) and PM were available for subsequent DGE separation. It follows from Fig. 11a and b that CCVs were in a region almost completely devoid of vesicular protein. Apart from CCVs proper (at fraction 56) clathrin-coated pits at the plasma membrane were detected (at fraction 46) as well as clathrin-coated structures, presumably residing on the transGolgi network (Tulp et al., manuscript submitted).

4. Conclusions and future prospects

For analytical purposes the future undoubtedly lies in high-performance CZE. It can be envisioned that on-and-off-rates of proteins contributing as building

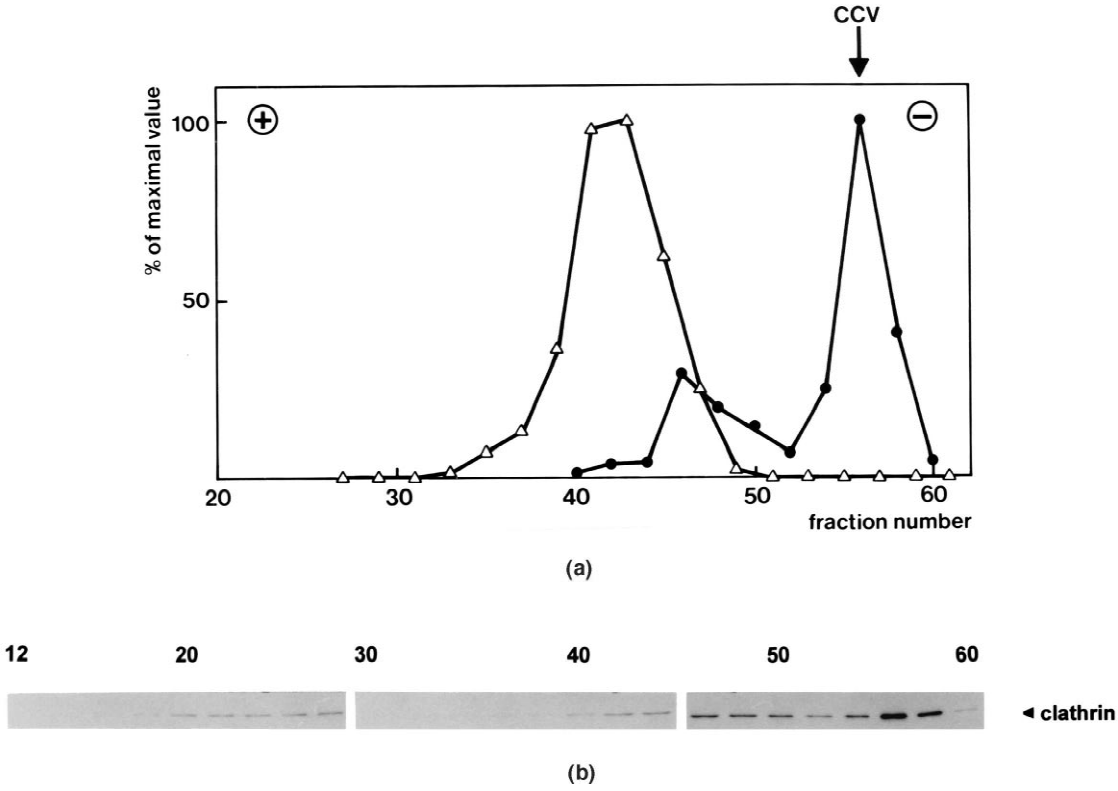


Fig. 11. Purification of clathrin-coated vesicles by DGE. (a) Organelles, derived from MelJuSo cells were first floated on a 0.25 *M*/0.86 *M* sucrose interphase using density gradient centrifugation. Organelles from the interphase were subjected to DGE as described in Ref. [53] for 80 min at 10.7 mA. (△–△) protein; (●–●) clathrin-coated vesicles (see b). (b) Immunoblot of clathrin. Numbers denote fraction number; fractions (from a) were subjected to SDS-PAGE and clathrin made visible using the Mab directed at the heavy chain. Note that three populations of clathrin were detected.

blocks to the complexes can be measured, provided that these association and dissociation rates are slower than the actual electrophoresis separation time. On the preparative side, both FFE and DGE are valuable to the purification of complexes. Such purified complexes can then be used for crystal formation leading to establishment of the quarternary structure. The authors are biased in this respect toward DGE since it is a simple, robust, inexpensive and rapid method.

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