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Chemical modification of proteins to improve the accuracy of their relative molecular mass determination by electrophoresis

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Abstract

We studied electrophoretic behavior of basic proteins (cytochrome c and histone III) and developed a carbamylation method that normalizes their electrophoretic size separation and improves the accuracy of their relative molecular mass determined electrophoretically. In capillary zone electrophoresis with cationic hitchhiking (CZECH), native cytochrome c does not sufficiently bind cationic surfactants due to electrostatic repulsion between the basic protein and cationic surfactant. Carbamylation suppresses strong positive charge of the basic proteins and results in more accurate relative molecular masses.

Keywords

Capillary sieving electrophoresis; Capillary zone electrophoresis with cationic hitchhiking; Carbamylation; Protein size separation; SDS capillary electrophoresis

1 Introduction

Size separations of protein by sieving electrophoresis in the presence of an ionic surfactant are efficient methods to separate proteins and determine their relative molecular mass (M_r). SDS polyacrylamide gel electrophoresis (PAGE) [1–3] has been a popular method for analysis of proteins for more than 40 years. Shortly after its introduction, a method separating proteins by PAGE in the presence of cationic surfactants was also presented [4]. When capillary electrophoresis was introduced, SDS PAGE was first modified for separations in capillary format with crosslinked polyacrylamide gel [5,6], numerous applications using various polymer solutions as a sieving medium followed [7–28]. Recently we developed a method of capillary sieving electrophoresis in the presence of a cationic surfactant and called it capillary zone electrophoresis with cationic hitchhiking (CZECH) [29].

Guttman and Nolan investigated the accuracy of relative molecular mass (M_r) accuracy of 65 proteins as obtained by SDS electrophoresis in capillary and slab gel format. Independently of the format, more than one fourth of proteins exhibited a biased migration [12].

A protein migration anomaly was observed already in the early years of SDS PAGE when lysozyme and ribonuclease A did not migrate according to their M_r [1,2,4]. It was speculated the anomalous proteins did not a) completely unfold, b) saturate with SDS [2]. Post-translational modifications of proteins, such as glycosylation, phosphorylation, and

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sulfonation, were identified as a possible source of deviations from the idealized migration of proteins [9]. It was also found that even a single substitution of a neutral amino acid in α -crystallines resulted in a changed mobility in SDS PAGE and thus in an erroneous M_r [30].

Glycoproteins were proposed to bind SDS less than other proteins since hydrophilic carbohydrates do not strongly interact with SDS. Deglycosylation of several glycoproteins with N-glycosidase F improved the accuracy of M_r of several glycoproteins in SDS PAGE and SDS CSE on microchip [31].

Electrostatic repulsion between ionic surfactants and proteins may be a major factor and cause of lower surfactant binding to proteins and consequently a biased migration [32]. This hypothesis was corroborated by an observation that some acidic proteins, such as pepsin, papain, and glucose oxidase did not bind a measurable amount of SDS [33]. Acidic ferredoxins, anomalously migrating in SDS PAGE, normalized their migration after esterification of their carboxyl groups with methanol [4]. The opposite effect was achieved when maleylation of cyanogen bromide fragments of cytochrome c was applied: it significantly reduced their apparent M_r in SDS PAGE while that of native cytochrome c was not much affected by carbamylation [34]. The falsely low M_r was obviously obtained because of high intrinsic charge of maleylated peptides and their electrostatic repulsion from SDS.

It was recommended to use the so-called Ferguson plot to correct M_r of proteins with biased migration [13,15]. The method improved the accuracy of M_r of some proteins but, e.g., for IgG L-chain, it still showed 17% error [12].

We have developed a method of protein carbamylation, which resulted in an improved accuracy of M_r as demonstrated on cytochrome c and histone III.

2 Materials and Methods

2.1. Materials

CZECH GelTM [35] and coated capillary iCapTM were obtained from Alcor BioSeparations LLC (Palo Alto, CA, USA). Tris(carboxyethyl)phosphine hydrochloride (TCEP) was a product of Gold Biotechnology (St. Louis, MO, USA). All other chemicals were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

2.2 Sample preparation

Protein samples were typically prepared by dissolving 5 g/L protein in 100 mM KCl, 2 g/L CTAC, and 10–50 mM TCEP or 100 mM dithiothreitol (DTT) and incubated at 70°C for 10 min.

2.3 Protein carbamylation

5 mg of a protein were dissolved in 820 μ L of water and mixed with 40 μ L of 25% m/v cetyltrimethylammonium chloride (CTAC), 40 μ L 10% m/v TCEP, and 100 μ L 1 M KCNO. After dissolving, the mixture was heated at 70°C for 20 min and then cooled to room temperature.

2.4 CZE of carbamylated cytochrome c

The CE experiments were performed on 3D CE capillary electrophoresis instrument (Agilent, Waldbronn, Germany). The carbamylated cytochrome c was analyzed by capillary zone electrophoresis in a coated capillary iCapTM, 50 μ m id, 360 μ m od, total length 335 mm, effective length 250 mm, in 100 mM β -alanine citrate, pH 3.3. Samples were injected

electrokinetically at 5 kV for 10 s and separated at +10 kV for 30 min. Cytochrome c and its derivatives were detected by measuring light absorption at 214 nm and 420 nm.

2.5 CZECH Separation

Capillary zone electrophoresis with cationic hitchhiking (CZECH) was performed in bare fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with 75 μm id and 360 μm od (the total capillary length of 335 mm and effective length of 250 mm). Between the runs, the capillary was flushed with 0.1 M citric acid, 0.1 M 2-hydroxyisobutyric acid (2-HIBA), or 0.1 M HCl, for 4 min and then the capillary was filled with the separation medium for 4 min. Proteins were injected electrokinetically at +8 kV for 15 s, separated at +10 kV for 20 min, and detected by UV absorption at 214 nm.

3 Results and Discussion

3.1 Cytochrome c carbamylation

Bovine heart cytochrome c (M_r 12,400) migrated in CZECH more slowly than expected, exhibiting the apparent M_r over 50,000. We conjectured it was caused by an insufficient interaction with cetyltrimethylammonium chloride (CTAC) during the denaturation as a result of a strong electrostatic repulsion. A modification that would suppress strong intrinsic positive charge of cytochrome c should result in a correction of the biased M_r . One of possible modifications is carbamylation, i.e., a reaction of lysyl primary amines with cyanate when homocitrulline is formed [36]

The course of cytochrome c carbamylation at room temperature was monitored by CZE at pH 3.3 in absence of any cationic surfactant and any sieving matrix (Fig. 1). Shortly after the initiation of the reaction, the native cytochrome c remained as the major component of the mixture, while only a small portion of cytochrome c was reacted. As the degree of carbamylation increased, the peaks became more numerous, slower and also broader. After 2 hours, there were several peaks corresponding to a partial cytochrome c modification. Native cytochrome c was still present in some extent in the reaction mixture. After 11.5 hours and particularly 17.2 hours, there was no detectable native cytochrome c. The electropherogram showed a number of peaks corresponding to different degree of carbamylation of various amino groups. Because bovine heart cytochrome c contains 19 lysyl residues [36] and none of them is 100% carbamylated, the reaction mixture contained a high number of various cytochrome c derivatives with different mobilities.

3.2 CZECH of carbamylated cytochrome c

Carbamylated cytochrome c represents a number of derivatives with a statistical distribution of unreacted and carbamylated amino groups. Denaturation kinetics of carbamylated cytochrome c in the presence of CTAC is a bit unusual: When carbamylated cytochrome c is simply injected without any heating, its apparent M_r is about 12,000, i. e., –2.9% less than the true M_r of cytochrome c. Incubating carbamylated cytochrome c at 95°C in the presence of CTAC resulted in discernable longer migration times in CZECH and so the apparent M_r of carbamylated cytochrome c increased and, after 10 min, achieved the value of 13,700 (Fig. 2). Interestingly, there is not only a difference in the migration times with the time of duration of denaturation but also the peak of carbamylated cytochrome c gets taller within the course of denaturation. Carbamylation of cytochrome c may only partly increase its absorbance at 214 nm because it is almost complete before the beginning of the denaturation so its contribution is probably not very significant.

In practical analysis, it would be preferred to perform both carbamylation and denaturation with CTAC in one step. In this case, 100 mM KCl should be simply replaced with 100 mM KCNO. Fig. 3 shows the time course of simultaneous carbamylation and heat denaturation at 70°C in the presence of CTAC. 5-min denaturation suffices to make cytochrome c migrate with correct mobility and M_r . Prolonged denaturation/carbamylation may result in a gradual increase of migration times and apparent M_r .

It is interesting to compare two basic proteins: lysozyme and cytochrome c. Both of them are likely to interact only weakly with CTAC, due to the electrostatic repulsion. In fact, this presumption is only true for cytochrome c while lysozyme is saturated with CTAC after an extended denaturation and thus migrates with correct M_r . Lysozyme contains 11 arginyl and 6 lysyl residues, cytochrome c 2 arginyl and 19 lysyl residues [37]. In other words, basic properties of lysozyme primarily stems from the presence of arginine, while cytochrome c contains mainly lysine. This suggests lysyl residues are more involved in electrostatic repulsion with CTAC than arginine and cause major errors in the determined M_r .

In CZECH, cytochrome c migrates with the velocity that corresponds to the apparent M_r of about 50,000 (Fig. 4). Carbamylation, which means reduction of positive charge of native cytochrome c, suppresses electrostatic repulsion between CTAC and cytochrome c, supports binding of CTAC to cytochrome c, and normalizes the electrophoretic migration of cytochrome c in CZECH and also resulting M_r (Fig. 5). This experiment confirms the hypothesis that electrostatic repulsion is the major cause of weaker interactions between proteins and cationic surfactants and thus of lower mobility of cytochrome c [32].

The carbamylation method was also applied to histone III with M_r 15,300. Denatured histone III exhibited M_r of 78,000 in CZECH, with the error over 400%. Carbamylated histone III migrated with a mobility corresponding to M_r of 15,500, i. e., with the error 1.3%.

3.3 Ferguson plot of carbamylated cytochrome c

Ferguson plot shows the effect of the concentration of a sieving matrix on the mobility of proteins. Typically, the Ferguson plot is represented by a straight line with a slope called retardation coefficient [38]. As the concentration of gel or sieving polymer increases and the pore size decreases, the migration of proteins is more retarded. At zero concentration of the sieving matrix, proteins exhibit the mobility in free electrolyte. Standard proteins behave properly and show adequately retarded migration with the increasing polymer concentration (Fig. 6).

However, when mobilities of denatured cytochrome c or any other protein with biased migration are plotted vs. the polymer concentration, a rather flat line is obtained. Cytochrome c migrates through the sieving matrix with a low velocity that almost remains unchanged through a relatively broad range of the polymer concentration. As pointed above, cytochrome c does not bind a standard amount of CTAC due to the electrostatic repulsion. It probably means that, even after denaturation, cytochrome c remains mostly folded and does not change much its native conformation. A question is the relationship between its size (Stokes radius) and the pore size of the sieving matrix at various polymer concentrations. Because of low M_r of cytochrome c, we can consider it to migrate in the Ogston rather than reptation mode [39]. In the Ogston mode, the electrophoretic retardation is primarily caused by collisions between the migrating protein and obstacles (polymer fibers). However, even in undiluted polymer, cytochrome c migrates with the mobility almost corresponding to free mobility. It probably does not mean the pores in concentrated polymer network are still much larger than the Stokes radius of cytochrome c. Carbamylated cytochrome c is probably less rigid structure than native cytochrome c. This should make the latter collide more with

the polymer fibers as the concentration of the polymer increases, i.e., it should result in the larger absolute value of its retardation coefficient. The denatured and carbamylated cytochrome c behave in reality in quite opposite way.

It was proposed to use Ferguson plot to determine correct M_r of proteins with anomalous migration [8,15]. Our data do not support this recommendation. As discussed above, proteins with anomalous migration including cytochrome c show rather flat Ferguson plot with low absolute value of retardation coefficient. When the retardation coefficient is used to calculate M_r , it provides acceptable results for normally migrating proteins but for the proteins with anomalous migration, it results in smaller apparent mobilities. E. g., while the carbamylated cytochrome c showed M_r of 12,300 as determined from the Ferguson plot, the denatured cytochrome c, exhibited the apparent M_r of about 300.

Although M_r of most proteins can be determined by MS, there is still a need for high-performance method separating proteins. Several years ago, protein separations were expected to be old-fashioned and capillary electrophoresis on microchip was viewed as a method just delivering proteins into MS where all analysis would be performed. This extreme opinion seems to be at least temporarily abandoned and MS is combined with SDS CSE [40]. On the other hand, electrophoretic and chromatographic size-separation methods are used less as a primary method for M_r determination.

4 Concluding Remarks

The accuracy of protein M_r determination can be improved by chemical modification of proteins that suppresses the electrostatic repulsion between ionic surfactants and proteins. However, the method is not a panacea for failed M_r determination. Similarly as in SDS PAGE and SDS CSE, there are still some recalcitrant proteins that migrate anomalously in CZECH and that provide biased M_r with an unacceptably high error.

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Abbreviations

CSE	capillary sieving electrophoresis
CTAC	cetyltrimethylammonium chloride
CZECH	capillary zone electrophoresis with cationic hitchhiking
GDH	glutamate dehydrogenase
2-HIBA	2-hydroxyisobutyric acid
TCEP	tris(carboxyethyl)phosphine hydrochloride

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Fig. 1. Time course of cytochrome c carbamylation as monitored by CZE
BGE: 100 mM β -alanine, 100 mM citric acid. Coated capillary: iCapTM (Alcor BioSeparations), total length= 335 mm, effective length= 250 mm, id= 50 μ m, od= 360 μ m. Voltage: +10 kV. Detection: UV absorption at 214 nm. Pressure injection: 3 s at 50 mbar. Sample: 5 g/L cytochrome c in 100 mM KCNO, reaction performed at room temperature.

Fig. 2. Carbamylated cytochrome c denatured for 0–10 min

Sample: 5 g/L cytochrome c carbamylated in 100 mM KCNO at room temperature, 10 g/L CTAC, 0.4% m/v TCEP, denatured at 95°C for 0–10 min. Separation medium: CZECH Gel™, bare fused silica capillary, total length= 335 mm, effective length= 250 mm, id= 50 μ m, od= 360 μ m.. Voltage: +10 kV. Electrokinetic injection: 15 s at +8 kV. Detection by UV absorption at 214 nm.

Fig. 3. Effect of simultaneous carbamylation and denaturation on migration of cytochrome c
Sample: 5 g/L cytochrome c in 100 mM KCNO, 10 g/L CTAC, 0.4% m/v TCEP, denatured at 70°C for 0–20 min. Other experimental conditions as in Fig. 2.

Fig. 4. Electropherogram of native and carbamylated cytochrome c in CZECH

Sample: carbamylated cytochrome c: 5 g/L cytochrome c in 10 g/L CTAC, 100 mM KCNO, 0.4% m/v TCEP, native cytochrome c: 5 g/L cytochrome c in 10 g/L CTAC, 100 mM KCl, 0.4% m/v TCEP,. Other separation conditions as in Fig. 2.

Fig. 5. Ferguson plot of native and carbamylated cytochrome c and standard proteins
 Proteins: + - lysozyme, O - β -lactoglobulin, Ψ - ovalbumin, $\diamond$ - glutamate dehydrogenase, Δ - BSA (monomer), $\times$ - phosphorylase b, $\square$ - BSA dimer, $\blacklozenge$ - cytochrome c, $\blacktriangle$ - carbamylated cytochrome c. Experimental conditions same as in Fig. 2.