Carbamylation of Proteins in 2-D ElectrophoresisMyth or Reality?

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Carbamylation of Proteins in 2-D Electrophoresis—Myth or Reality?

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Carbamylation is widely quoted as being a problem in 2-D gel analysis and the associated sample preparation steps. This modification occurs when iso-cyanate, a urea break-down product, covalently modifies lysine residues, thus inducing a change in isoelectric point. Urea is used at up to 9 M concentrations in sample preparation and 2-D gels because of its ability to disrupt protein structure and effect denaturation without the need for ionic surfactants such as SDS. We have studied carbamylation using 7 M urea and 2 M thiourea, under a range of experimental temperatures to establish when, and if, it occurs and what can be done to minimize the modification. The actual time required for protein extraction from a tissue is usually short compared to the time required for procedures such as reduction and alkylation and IPG rehydration and focusing. Therefore, it is the temperature during these post-extraction procedures that is the most critical factor. Our experiments have shown that carbamylation does not occur during electrophoresis in the presence of urea, even with prolonged run-times. However, under poorly controlled sample preparation and storage conditions, it can become a major event.

Keywords: two-dimensional electrophoresis • proteomics • carbamylation • artifacts • reduction • alkylation • MALDI-TOF mass spectrometry

1. Introduction

Proteomics demands high resolution separation techniques to penetrate the complexity of even the simplest organisms. The relatively small number of genes in the human genome has reinforced the major role of co- and post-translational modifications in generating complexity. Given this situation, one of the most fundamental requirements in protein separations is that the analytical technique does not further modify the sample during analysis. Two-dimensional (2-D) electrophoresis remains the highest resolution separation method; however, it has been tarnished by suggestions of artifactual modifications induced during the process, with carbamylation being among the most frequently quoted offenders.

The preparation of proteins for denaturing 2-D electrophoresis requires the use of chaotropic agents to ensure that protein complexes are disrupted and individual polypeptides are unfolded. Chaotropic agents disrupt hydrogen bonding and cause protein unfolding, thus exposing hydrophobic amino acid residues to the solution. For this reason, nonionic or zwitterionic surfactants such as CHAPS (3-[(3-cholamido-propyl)-dimethyl-ammonio]-1-propane sulfonate) are used in combination with chaotropes to coat the hydrophobic residues and improve solubility.[1] Urea is the most commonly used chaotropic agent and, increasingly, thiourea/urea combinations are used to exploit the improved denaturing ability of thiourea.[2] In solution, urea is in equilibrium with ammonium cyanate as shown in Figure 1a.[3] Using mixed-bed ion exchangers, urea solutions can be prepared that are initially cyanate free, but the concentration of ammonium cyanate will slowly increase over time until equilibrium is reached. However, if a cyanate

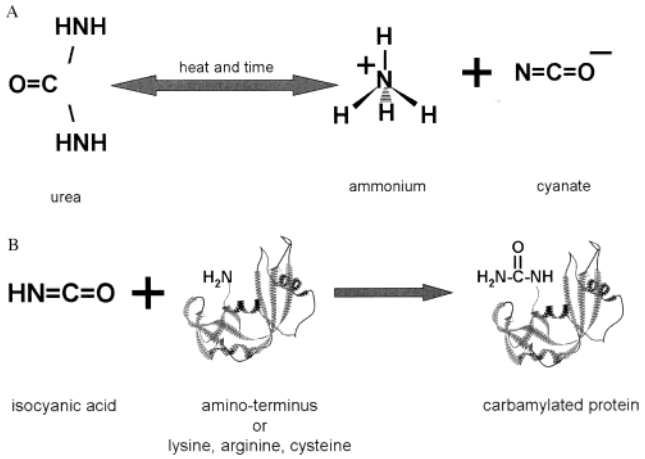

Figure 1. Scheme of urea degradation and its action on proteins. (A) degradation path, driven by heat, time and pH; (B) attack of isocyanic acid onto reactive groups in proteins.

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scavenger, such as the ϵ-amino group of lysine, is present the formation of cyanate will continue unabated until the scavenger is completely consumed. At temperatures below 37 °C, the degradation of urea proceeds slowly and concentrations of cyanate do not reach problematic levels within the time of most sample preparation prodecures. Higher temperatures accelerate the rate at which ammonium cyanate is produced and thus should be avoided when preparing protein samples in urea. Cyanate has been shown to react with nucleophilic groups such as the protein amino terminus, the amino side chains of lysine and arginine residues, and the sulfhydryl group of cysteine residues[4−6] as shown in Figure 1b. The reaction occurs more rapidly in alkaline conditions when the nucleophilic groups are deprotonated and thus more reactive and the relative reactivity of the residues is dependent on their pK_a. The free base forms of aliphatic amines, such as the ϵ-amino group of lysine, are present at very low concentrations below pH 8. The carbamylation reaction of amines by isocyanic acid is strongly pH dependent and a pH of 8.5−9.5 is usually optimal for modifying lysine residues. In contrast, the α-amino group at a protein's amino terminus is neutral, with a pK_a of ∼7, and may be selectively modifed by reaction at near neutral pH.[7] The carbamylation modification results in an increase of 43 AMU relative to the unmodified protein or peptide.[8]

There is increasing awareness of the need for prefractionation to reduce the complexity of samples in proteome analysis. This has led a number of groups to investigate various methods of fractionation and their compatibility with downstream 2-D gel analysis, especially on narrow range pH gradients. With the introduction of immobilized pH gradients, it was demonstrated that IEF in narrow pH ranges could separate proteins that differed by p*I* values of 0.01 to 0.001 pH units.[9] With complex samples only one technique, isoelectric fractionation, is ideal for prefractionation prior to 2-D on narrow range IPGs.[10] MCE fractionation is currently the most suitable method for 2-D gel sample preparation because it is a form of IEF and the fractions are totally compatible with subsequent 2-D separations. The potential drawback of MCE fractionation is that it exposes the protein to denaturing conditions, in high urea concentrations, for longer than 2-D alone. When combining MCE and 2-D, the focusing times, and thus urea exposure, can approach 48 h, with temperatures during uncooled MCE operations reaching 40 °C. To investigate if proteins would be carbamylated under these conditions, we trapped a urea denatured peptide in the MCE under alkaline conditions and investigated potential modifications using linear mode MALDI MS. In addition, to highlight the potential dangers of poor sample storage and extraction techniques we prepared an extract of *Escherichia coli* and stored it at different temperatures.

2. Materials and Methods

2.1 Materials. Urea, tributyl-phosphine (TBP), acrylamido buffers, trifluoroacetic acid (TFA), and sodium dodecyl sulfate (SDS) were obtained from Fluka Chemie (Buchs, Switzerland). ProteoPrep kits (Prot-Tot), myoglobin, cyanogen bromide, Tris-(hydroxymethyl)aminomethane, *E. coli* K12, Coomassie Brilliant Blue G, ammonium sulfate, methanol and phosphoric acid, were from Sigma (St. Louis, Missouri).

2.2 Sample Preparation. Dry *E.coli* cells (3 mg/mL) were solubilized by sonication in Sigma ProteoPrep (Prot-Tot) extraction solution No.4, pH 10.4. To minimize the formation of cyanate, it is important to maintain the sample below 35 °C during the sonication. The solution was kept in on ice and sonicated for 4 bursts of 30 s with 1 min cooling between bursts. After centrifugation at 40 000 g for 20 min, the supernatant was reduced with TBP (5 mM) and alkylated with 15 mM iodoacetamide (IAM)[11,12,13] prior to storage at either −20 °C, 20 °C, 30 °C, or 50 °C.

To prepare an alkaline low-mass peptide, myoglobin (horse skeletal muscle) (20 mg/mL) was digested with CNBr (10 mg/mL) in 50% TFA, overnight at room temperature. The digested myoglobin was diluted 10-fold with MilliQ water and dried by vacuum centrifugation. The efficiency of the CNBr cleavage was determined by nano-electrospray-MS on a Thermo Finnigan LCQ Deca XP Instrument (Thermo Finnigan, San Jose, CA). The vacuum-dried myoglobin peptide was solubilized (0.1 mg/mL) in 0.1% (v/v) TFA and purified using a C18 ZipTip (Millipore, Boston, MA) with elution in 50% acetonitrile, 0.1% (v/v) formic acid. The myoglobin peptide was desalted on a C8 column and dried by vacuum centrifugation.

2.3 Multicompartment Electrolyzer Fractionation. A sample of the myoglobin peptide was solubilized in 8M urea and focused in an MCE with four chambers for 48 h. The chambers of the MCE were separated by acrylamide/Immobiline membranes (7.5%T, 10%C) cast onto glass fiber disks (2 mm × 24 mm, pore size 2.7 μm). The buffering disks had pH values of 3.0, 8.0 and 10.0. The concentrations of acrylamido buffers for each membrane were determined by the Doctor pH software package (Hoefer, San Francisco, CA). The myoglobin peptide was loaded into the alkaline pH 8.0−11.0 chamber of the MCE. Focusing was for 4 h at 100 V followed by 44 h at constant 1 W and temperature was monitored regularly using a thermocouple.

A control sample was solubilized in 8 M urea, sodium borate pH 9.0, and maintained at an equivalent temperature to the MCE sample for 48 h.

Conductivity measurements of the MCE chamber solutions and the control sample were regularly made using a microconductivity meter.

2.4 2-D Electrophoresis. The *E. coli* extracts were loaded by rehydration (250 μL) onto 11 cm pH 3−10 ProteoGel IPG strips from Sigma (St Louis, MO). IEF was conducted using a 5 h linear ramp to 10 kV and a further 5 h at 10 kV, using a prototype IEF device under development at Proteome Systems. Second dimension gels were GelChip long life tricine chemistry gels available from Proteome Systems (Woburn, MA) or Sigma (St. Louis, MO). Gels were stained using colloidal Coomassie Brilliant Blue G250 according to the method of Neuhoff [[14]].

2.5 MALDI-TOF MS. Samples were loaded onto the target plate using the "three-layers" method: 0.6 μL of matrix solution [10 mg/mL sinapinic acid in 40% (v/v) acetonitrile, 0.1% (v/v) TFA] (prepared fresh every day) were loaded onto the sample plate and left to dry at room temperature. One μL of sample solution was deposited on the matrix layer and left to dry and then covered with 0.6 μL of the matrix solution. MS was performed using a MALDI-TOF Axima CFR mass spectrometer (Kratos, Manchester, UK), equipped with a pulsed nitrogen laser (337 nm, pulse width 4 ns) and operated in delayed extraction mode with an acceleration voltage of 25 kV. Spectra were recorded in linear mode for intact mass peptide analysis.

3. Results and Discussion

3.1 *E. coli* Extract 2-D Gels. The effect of carbamylation, as observed on 2-D gels, is the incremental acidic shift of

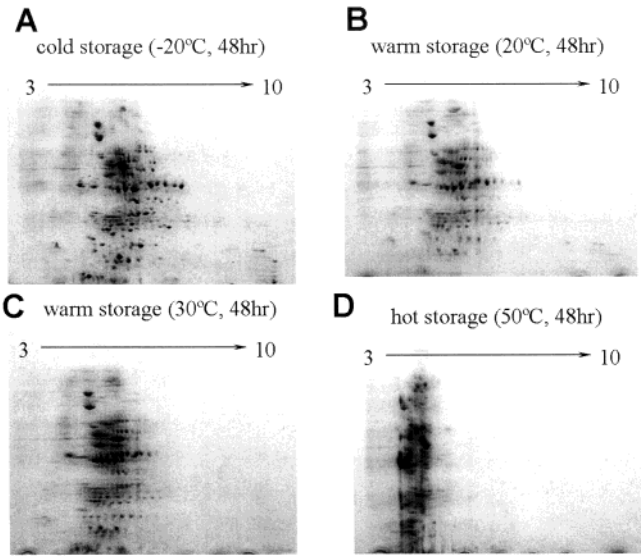

Figure 2. Two-dimensional map analysis of *E. coli* extracts incubated under various conditions in 8 M urea. Note how long exposures at relative high temperatures (50 °C) induces large scale carbamylation of the whole protein sample, with a dramatic shift of all the train of spots in the acid pH region (lower right panel).

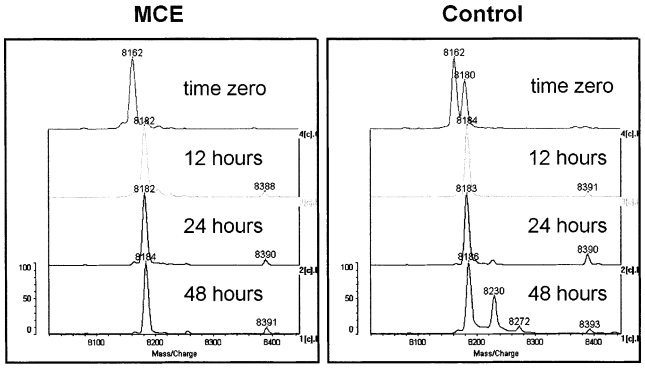

Figure 3. Extent of carbamylation of a myoglobin peptide in the presence of 8 M urea in the electric field (left panel) and in a test tube standing on a bench (right panel). Analysis by MALDI-TOF MS. Note the absence of carbamylation in the sample kept under a voltage gradient.

isoelectric point as positive charges are lost as a result of amino group modification. This is clearly shown in Figure 2: as the temperature of storage increases, the alkaline proteins are lost until in the 50 °C sample, there is only a vertical band of proteins compressed at the acidic end of the gel. In the early stages of carbamylation, or under typical room-temperature storage, the effect is often observed as the emergence of strings of spots with p*I*'s more acidic than the original "parent" spot (Figure 2B and C). A shift in mass is not observed as the addition of 43 mass units as a result of carbamylation is beyond the resolving power of SDS−PAGE. The *E. coli* extracts were stored at alkaline pH, a prerequisite of the reduction and alkylation performed to minimize artifactual spot appearance as a result of disulfide reformation.[11−13] The ProteoPrep solubilization cocktail contains 40 mM Tris to ensure that the pH is above 9 and ideal for complete cysteine alkylation. The drawback of alkaline pH is that, in the presence of urea, it is also ideal for carbamylation. One could titrate the pH to a neutral value to minimize the chance of carbamylation, but the amount of acid required to titrate 40 mM Tris would probably overload the IPG with ionic species and disturb the focusing. Our recommendation is to minimize extraction time and ensure that the sample is kept as cool as possible during extraction and subsequent storage without causing urea precipitation and at least below 37 °C at all times. Storage of urea containing extracts should always be at 4 °C for short periods and frozen for long-term storage.

3.2 MCE Fractionation. The kinetics of carbamylation should be very different in an electric field compared to the situation described above. During electrophoresis, the charged products of urea degradation should be rapidly transported to the electrodes thus providing minimal opportunity to react with amino groups on proteins and peptides. The protective effect of the electric field is shown in Figure 3, where the left panel shows MS analysis of the myoglobin peptide trapped in the MCE and the right panel is the control with no electric field. The mass increase from 8162 to 8180 observed on both MCE and control peptides is the conversion of the terminal methionine to homoserine lactone. In the 24 and 48 h control samples, carbamylation is clearly observed as the appearance of peaks with masses of 8230 and 8272, being one and two carbamylation events, respectively. No peaks corresponding to the addition of 43 AMU were observed in the MCE samples even after 48 h of trapping in the alkaline chamber. As the only buffering species present, the pH of the alkaline MCE chamber would be close to 9.3, defined by the calculated p*I* of the peptide. The additional masses observed at 8388 to 8393 are unidentified contaminants.

To further explore the conditions present within the MCE, we measured temperature and conductivity throughout the course of the experiments and plotted the results. The temperature and conductivity graphs in Figure 4 clearly illustrate the low conductivity that is maintained in the sample chambers of the MCE during the course of the focusing. The lower line is the conductivity curve in each of the graphs. It is easy to observe the rapid transport of cyanate ions present in the sample chambers to the anodic reservoir, thus maintaining a cyanate-free environment during the run. This might not apply to the ammonium ion, a weak ion with a p*K* of 9.25. At the prevailing pH in this chamber, it will be about 50% deprotonated, so that its transport toward the cathode will occur at a reduced rate. It is also possible that, upon crossing the pH 11 membrane, it will become rapidly deprotonated, so that, ultimately, its transport into the cathodic compartment will be substantially diffusion-driven. Nevertheless, in a urease reactor based on the MCE principle, the passage of ammonium ions through a pH 11 membrane and its accumulation into the cathodic compartment has been well documented, both theoretically and experimentally.[15] In addition, a longer residence time of the ammonium ion in this chamber would hardly affect the carbamylation event, were it to occur.

It is also interesting to note that the (rather modest) build-up of ions and concomitant increase in conductivity in the electrode chambers does not begin until the 4 h mark, when the input power is increased to 1 W, thus increasing the temperature of the solutions. Figure 5 shows a comparison between the conductivity of a control peptide sample and the alkaline MCE chamber. The conductivity in the control sample rises for the duration of the experiment, whereas the MCE sample maintains very low conductivity.

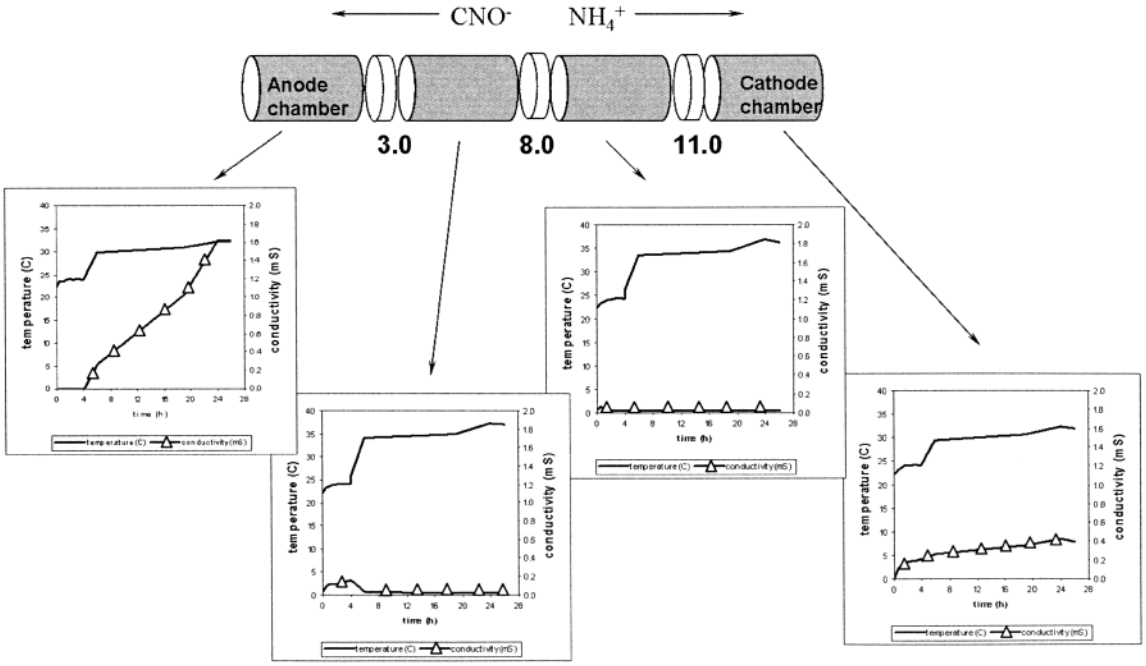

Figure 4. Schematic diagram of the fractionation in the multicompartment electrolyzer. The four lower panels refer to temperature (upper curve) and conductivity (lower curve) in the four chambers of the apparatus. Note the constant increment of conductivity with time in the anodic chamber, probably due to cyanate accumulation, and the relatively modest increments in the cathodic chamber, probably due to deprotonation of the ammonia ions, the two central sample chambers exhibiting, in contrast, a negligible conductivity.

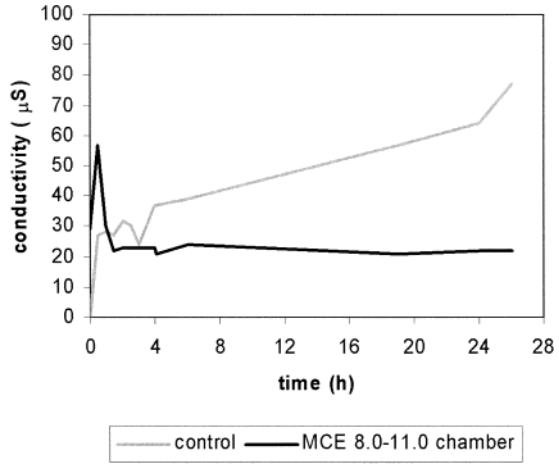

Figure 5. Variations of conductivity in the control sample (in the absence of electric field; upper curve) and in the MCE device under the electric field (lower curve).

4. Conclusions

Proteomics continues to grow and assume an important role in the biological research programs of many organizations. The technology used for proteomics is therefore under ever increasing scrutiny, which is why it is important to lay another myth to rest here. By a combination of careful sample preparation and the protective effect of the electric field during electrophoresis it is possible to banish carbamylation from 2-D maps forever.

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