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Native capillary isoelectric focusing for the separation of protein complex isoforms and subcomplexes

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Abstract

Here we report the use of capillary isoelectric focusing under native conditions for the separation of protein complex isoforms and subcomplexes. Using biologically relevant HIS-tag and FLAG-tag purified protein complexes, we demonstrate the separations of protein complex isoforms of the mammalian target of rapamycin complex (mTORC1 and 2) and the subcomplexes and different phosphorylation states of the Dam1 complex. The high efficiency capillary isoelectric focusing separation allowed for resolution of protein complexes and subcomplexes similar in size and biochemical composition. By performing separations with native buffers and reduced temperature (15°C) we were able to maintain the complex integrity of the more thermolabile mTORC2 during isoelectric focusing and detection (< 45 min). Increasing the separation temperature allowed us to monitor dissociation of the Dam1 complex into its subcomplexes (25°C) and eventually its individual protein components (30°C). The separation of two different phosphorylation states of the Dam1 complex, generated from an *in vitro* kinase assay with Mps1 kinase, was straightforward due to the large pI shift upon multiple phosphorylation events. The separation of the protein complex isoforms of mTORC, on the other hand, required the addition of a small pI range (4 – 6.5) of ampholytes to improve resolution and stability of the complexes. We show that native capillary isoelectric focusing is a powerful method for the difficult separations of large, similar, unstable protein complexes. This method shows potential for differentiation of protein complex isoform and subcomplex compositions, post-translational modifications, architectures, stabilities, equilibria, and relative abundances under biologically relevant conditions.

Protein assemblies are well known to make up the functional machinery of the cell.¹ What is less well understood is the dynamic nature of the protein interactions necessary for the biological machinery to function properly. Many diseases have been found to be caused by aberrant protein-protein interactions.² New techniques, and even fields, are emerging to better probe and understand protein-protein interactions within the context of a cell. Interactomics is a recently introduced subset of systems biology which focuses on the interactions of proteins and other molecules.³ For example, yeast two hybrid (Y2H) screens have generated binary protein interaction data for *S. cerevisiae*^{4, 5} and *C. elegans*.^{6, 7} Two global tandem-affinity purification (TAP) efforts in *S. cerevisiae* used mass spectrometry to

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identify proteins within purified complexes. The results increased the known curated complexes (217) from the Munich Information Center for Protein Sequences (MIPS) by 257⁸ and 275.⁹ Other efforts to investigate protein-protein interactions use native variations of common orthogonal biochemical separations of cell lysates to fractionate protein complexes for mass spectrometry (MS)-based analysis. Two-dimensional blue native polyacrylamide gel electrophoresis (BN-PAGE) allows for the elucidation of membrane bound protein complexes.¹⁰ More recently, successive preparative liquid chromatography separations facilitated unbiased identification of 13 known *E. coli* complexes¹¹ and 20 known *P. furiosus* complexes.¹² On the analytical scale, capillary electrophoresis (CE)-MS permitted separation and detection of three protein complexes directly from a cell lysate with a concentration dynamic range of ~3.¹³ Direct analysis of large (~50–700 kD), purified protein complexes in the gas phase is also progressing through instrumental and operational modifications to mass spectrometers.^{14–17} As a result, native mass spectrometry is quickly advancing as a method to elucidate protein complex composition,^{18–20} structure,²¹ and dynamics²² of purified protein complexes.

Experiments studying protein-protein interactions globally within the cell indicate the growing need for methods to address dynamic and versatile protein-protein interactions through direct physical or chemical analyses. Protein interactions are highly dependent on developmental, environmental, and genetic conditions,²³ making many proteins versatile in function, yet still highly specific. For instance, a single protein can differentiate cellular functions through participation in multiple protein complexes with distinct binding partners as shown in figure 1A. Protein complexes of this nature have been deemed protein complex isoforms.⁸ Further promiscuity of proteins is possible through participation in protein subcomplexes.²⁴ Subcomplexes are stable protein complexes within a larger protein complex as shown in figure 1A. Elucidation of protein complex isoforms and subcomplexes can be convoluted and arduous using conventional methods. Identification of protein complexes and differentiation of their isoforms and subcomplexes usually begins with co-purifications of known and suspected binding partners under native conditions. Potential binding partners are validated by identification via mass spectrometry-based proteomics²⁵ or western blotting.²⁶ An automated method for distinguishing these subtle differences would be highly beneficial to identify complex isoforms and subcomplexes directly.

From the separation science perspective, resolving protein complex isoforms and subcomplexes is challenging. Efficient protein separations are already difficult due to the inherent hydrophobicity and large size of proteins²⁷ and can suffer from recovery problems on the analytical scale.^{28–31} Well-studied protein complexes were found to contain 10 or more interacting proteins¹ and a global study in yeast confirmed an average of 12 proteins per complex.³² Larger protein complex assemblies only magnify the challenges associated with protein separations. A factor of 10 increase in analyte size results in an approximate order of magnitude reduction in diffusion coefficient and electrophoretic mobility; these are respectively detrimental to efficient separations using either liquid chromatography or electrophoretic separation methods. Additionally, by definition protein complex isoform compositions may only differ by a few proteins.⁸ With a majority of the proteins conserved between complex isoforms, the size, shape, charge, chemical composition, and thermodynamic character of complex isoforms may be relatively similar. A specific protein complex isoform comparison, based on size and isoelectric point, is illustrated in table 3. To separate complex isoforms, an extremely efficient, high resolution separation technique is required that can accentuate their subtle physical or chemical differences. Finally and most importantly, the technique must use conditions that can maintain biochemical and thermodynamic stability of the complexes.

Capillary isoelectric focusing (CIEF) has proven to be the best method for the analysis of protein isoforms, with the capability to distinguish proteins differing by as few as 0.005 pH units.³³ Proteins are focused to their isoelectric point in a capillary with a pH gradient generated by zwitterionic ampholytes and the opposing migration of an acid and base across an electric field.³⁴ The parallel between the subtle chemical differences of protein isoforms and protein complex isoforms made it likely that exploiting isoelectric point differences would also prove useful for separation of protein complex isoforms and subcomplexes. CIEF has become increasingly used for analysis of biochemical interactions such as protein-antibody,^{35–37} protein-ligand,^{38, 39} protein-DNA,⁴⁰ protein-phospholipid,⁴¹ and protein-drug^{42–45} complexes indicating the potential for elucidation of biologically relevant protein complexes. Identification of protein complex standards by Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS)⁴⁶ and molecular weight profiling of *E. coli* protein complexes⁴⁷ has also been previously demonstrated using CIEF. We will demonstrate herein that performing CIEF under native conditions allows for the separation and relative quantitation of protein complex isoforms and subcomplexes.

METHODS AND MATERIALS

Reagents and chemicals

Unless otherwise noted all chemicals were purchased from Thermo Fisher Scientific (Waltham, MA). Deionized water (18.2 M Ω , Barnstead, Dubuque, IA) was used for all preparations. The CIEF kit containing coated capillaries, pI 3–10 ampholytes, focusing acid and base, mobilization acid, anodic and cathodic stabilizers, and pI markers was obtained from Beckman Coulter (Brea, CA). Pharmalyte pI 4 – 6.5 ampholytes were purchased from GE Healthcare (Piscataway, NJ).

Dam1 Complex preparation

Saccharomyces cerevisiae Dam1 complex was expressed in and purified from *E. coli* as described previously.⁴⁸ Dam1 complex polycistronic vector was transformed into BL21 Rosetta (Novagen, Madison, WI). Cultures were grown to about 30 Klett units, and the cultures were induced overnight at 23°C. Pellets were lysed using a French press in 50 mM sodium phosphate buffer (pH 6.9) containing 350 mM NaCl and protease inhibitors (0.01 mg ml⁻¹ chymostatin, 0.01 mg ml⁻¹ aprotinin, 0.01 mg ml⁻¹ leupeptin, 0.01 mg ml⁻¹ pepstatin, 0.002 mg ml⁻¹ benzamide and 1mM phenylmethylsulfonyl fluoride). The Dam1 complex was purified by affinity chromatography using talon resin, according to the manufacturer's instructions (BD Biosciences, San Jose, CA). Peak elutions were concentrated to approximately 1 ml using a 50 kDa molecular weight cutoff Amicon Ultra centrifugal filter (Millipore, Billerica, MA) and then subjected to gel filtration chromatography on an SD \times 200 16/60 column (GE Healthcare). Peak fractions were concentrated and cleared at 13,000g. Glycerol (10%, final concentration) was added and aliquots were snap frozen and stored at -80°C.

Mps1 preparation and kinase assay

Saccharomyces cerevisiae Mps1 kinase was expressed in *E. coli* with a GST tag on the N-terminus and a 6XHis tag on the C-terminus from plasmid pDG54, (derived from plasmid pGEX6p-MPS1).⁴⁹ Expression was induced in 1 L of cells by addition of 40 mg IPTG and cells were grown overnight at 18 C. Mps1 was purified by the 6XHis tag using talon resin according to the manufacturer's instructions with the following exceptions. The cells were resuspended in 20 mM HEPES buffer, pH 7.2, containing 150 mM NaCl, 2% Triton X-100, and protease inhibitors and lysed using a French press. The protein was eluted from the column in 1.5 ml of 20 mM HEPES buffer, pH 7.2, containing 150 mM NaCl and 400 mM imidazole.

Dam1 complex was phosphorylated in 50 mM HEPES buffer, pH 7.2, containing 25 mM MgCl₂, 10 mM ATP, 150 mM NaCl, 4 μM Dam1 complex and 15 μl Mps1 kinase in a 25 μl reaction volume. The reaction was incubated at 30 C for 90 min. The stoichiometry of phosphorylation was determined under the same assay conditions except that gamma-³²P ATP (3000 Ci/mmol) was included at 0.5 μCi/μl.

mTOR complex preparation

To produce a soluble mixture of mTORC1 and mTORC2, we generated a HEK-293T cell line that stably expresses N-terminally FLAG-tagged mLST8 using vesicular stomatitis virus G-pseudotyped MSCV retrovirus. mTOR complexes were purified by lysing cells in 50 mM HEPES, pH 7.4, 200 mM NaCl and 0.4% CHAPS. Cells were lysed at 4°C for 30 min, and the insoluble fraction was removed by centrifugation at 18,000 rpm for 30 min. Supernatants were incubated with FLAG-M2 monoclonal antibody-agarose for 1 hr and then washed with three column volumes of wash buffer (50 mM HEPES, pH 7.4, 200 mM NaCl, 2 mM DTT and 2 mM ATP and 0.1% CHAPS). Purified mTOR complexes were eluted with 100 μg/ml 3x FLAG peptide in 50 mM HEPES, pH 7.4, 500 mM NaCl and 0.1% CHAPS. Eluted fractions were pooled and concentrated by centrifugation prior to CIEF separation. Samples were subsequently analyzed by SDS-PAGE and immunoblotting to confirm the purification of the complexes.

CIEF separations

Separations were performed on a PA800 Capillary Electrophoresis System (Beckman Coulter, Brea, CA) using a 50 μm ID/360 μm OD neutral coated capillary or a 100 μm ID/360 μm OD in-house hydroxypropyl cellulose (average molecular weight 100,000 g/mol) coated capillary⁵⁰ cut to 32 cm (20.2 cm to detector). The capillary was rinsed with DI water before and after runs at 50 psi for 2 min. All samples were prepared on ice and stored at 4°C prior to loading onto the capillary. The CIEF buffer was prepared to contain 1.7% w/v Pharmalyte pI 3–10 carrier ampholytes, 2.1% w/v Pharmalyte pI 4 – 6.5 carrier ampholytes (for mTORC separation only), 42 mM arginine, and 1.7 mM iminodiacetic acid.⁵¹ Protein complexes were added to the prepared CIEF buffer immediately before analysis unless otherwise stated. The CIEF capillary was thermostated to 15°C except for protein complex stability experiments with Dam1 where separation temperatures of 20°C, 25°C, and 30°C were also used. Samples were loaded onto the capillary for 1 min at 50 psi. Isoelectric focusing was performed for 10 or 15 min at 25 kV with a maximum current of 50 μA using 200 mM H₃PO₄ and 300 mM NaOH at the anode (inlet) and cathode (outlet), respectively. High efficiency isotachophoretic mobilization of protein complexes past the detector after focusing was performed by substitution of 300 mM NaOH with 350 mM acetic acid at the cathode. Detection of mobilized protein complexes was performed at 4 Hz using a UV detector at 280 nm through a 200 μm aperture.

Viscosity correction for native CIEF separations at varying temperatures—To clarify the appearance or disappearance of peaks at different separation temperatures, we normalized the mobilization time of the higher temperature separations to the lowest separation temperature (15°C) using a viscosity correction. The correction was necessary since the viscosity of the CIEF buffer decreased with increasing temperature and caused a systematic decrease in the mobilization times of focused protein complexes illustrated in figure 3A. Corrections for viscosity-induced mobility shifts from buffer additives in capillary electrophoresis have been made using a viscosity correction factor based on absolute⁵² and relative viscosity measurements and separation currents.⁵³ We extended this to temperature-induced viscosity changes during CIEF. The viscosity correction based on migration time was possible since one large peak remained relatively constant throughout the separations, marked with an (*) in all separations in figure 3. These peaks were used as a

mobilization time marker for measurement of relative viscosity. Since the peak used for correction was not a spiked standard we also investigated mobilization current as a measure of relative viscosity. The equation used to calculate the viscosity correction factor based on mobilization time (v_t) is:

$$v_t = \frac{t_{15^\circ\text{C}}}{t_{\circ\text{C}}} \quad (1)$$

and based on current (v_I) is:

$$v_I = \frac{I_{15^\circ\text{C}}}{I_{\circ\text{C}}} \quad (2)$$

where t is the mobilization time of the peak and I is the average mobilization current at either 15°C or the higher temperatures, respectively. The mobilization time and current corrections are listed in table 2. There was close agreement between the values from the two correction methods, but we used v_t values since there is greater variability in the CIEF current relative to CE. The corrections were performed by multiplying the original mobilization times of the 20°C, 25°C, and 30°C separations by their corresponding viscosity correction factor. The viscosity-corrected separations are shown in figure 3B. Aligned peaks at different separation temperatures resulting from the viscosity correction are highlighted with red, blue, and black boxes.

Ω plot generation

For measurement of currents during the focusing step, CIEF buffer was loaded, voltage was applied for 1 min, the maximum current was measured, and the process was repeated for each voltage. For measurement of currents during the mobilization step, CIEF buffer was loaded, a 25 kV focusing voltage was applied for 10 min, the capillary outlet was switched to 350 mM acetic acid, a 30 kV mobilization voltage was applied for 30 min, and measurements were taken for 15 sec at each voltage. All measurements were made with the sample thermostated at 4°C and the capillary at 15°C.

RESULTS AND DISCUSSION

Implications of protein complex purification methods

In recent years biological studies have emphasized the identification and analysis of protein complexes involved in physiological processes. Methods and strategies have evolved, for example, for the large-scale analysis of protein complexes from different model organisms. Commonly, protein complexes are isolated using genetically integrated purification tags or antibodies,⁵⁴ as illustrated in figure 1A. The two complexes analyzed herein were purified using the His-tagged Spc34 protein from *E. coli* (Dam1 complex) and the FLAG-tagged mLST8 from HEK-293T cells (mTORC1 and 2). For comparison, most native mass spectrometry experiments of complexes have been performed on over-expressed proteins from *E. coli* with well know purification strategies.^{15, 16, 21} Recent advances in stabilization of soluble^{17, 55} and membrane bound^{56, 57} protein complexes are quite promising, yet it is likely there will always be protein complexes which will not be compatible with native mass spectrometry. Isolation of an endogenous protein complex using a particular protein as “bait” will yield a variety of protein isoforms or subcomplexes to which the protein belongs. To sort out the physiological roles of each of the protein complex forms and subcomplexes, methods to purify and study the complexes are needed. The versatility of native CIEF,

similar to MudPIT analysis of protein complexes,²⁵ should complement other methods for protein complex analysis.

Separation temperature control during native CIEF

Essential to protein complex isoform separation and analysis is maintenance of protein complex integrity prior to detection of the separated complexes. Traditional biochemical separations and purifications of protein complexes are often performed at 4°C to maintain the complex integrity and stability.²⁶ Our current instrument configuration limited capillary cooling to 15°C. To properly thermostat the capillary during separations even with active cooling, Joule heating must be avoided. Joule heating in capillary electrophoresis is the resistive heating of buffer in the capillary by the current generated from the high voltages applied during the separation. Joule heating is best known for its adverse effects on separation efficiency,⁵⁸ but can have many other detrimental effects.⁵⁹ In this case, we were concerned that internal heating of the buffer may cause dissociation of native complexes. Often this is not an issue with CIEF since viscosity increasing additives decrease the current and small inner diameter capillaries adequately disperse heat.^{51, 60} We eliminated viscosity additives for native conditions and tested both 50 and 100 μm inner diameter capillaries in the interest of preparative fractionation. The highest voltage in capillary isoelectric focusing yields the best isoelectric point resolution,⁶¹ so we determined the conditions which yielded the best possibility for resolving protein complex isoforms and subcomplexes. We tested the acceptable voltages and currents for focusing and mobilization because the current varies dramatically during CIEF runs, particularly with cathodic mobilization.⁶² As shown in figure 2A, the maximum voltages during CIEF occur at the beginning of the focusing step and the end of the mobilization step. Plotting the current generated at different voltages yields an Ω plot. A positive inflection from linearity indicates the onset of Joule heating conditions. Figure 2B illustrates that Joule heating occurs during the focusing at a voltage of 25 kV and current of 50 μA . Thus, 50 μA was set as the maximum current allowable for the two step process.

Protein complex stability during native CIEF

Little is known about the stability of protein complexes during CIEF. CIEF profiling of *E. coli* protein complexes using UV detection indicated a few unknown ~550 kDa protein complexes were stable during the course of separations, but not after 8 hrs.⁴⁷ Thus, we investigated the effects of separation temperature on the stability of a known protein complex, Dam1. The 10-mer Dam1 complex is part of the outer kinetochore in yeast, an arrangement of at least 65 proteins, which is responsible for attachment of sister chromatids to microtubules for segregation during mitosis.⁶³ Phosphorylation of the protein Dam1 within the complex by Mps1 kinase is required for coupling of the kinetochore to the plus-ends of microtubules.⁶⁴ The purification and kinase assay developed for characterization of the Dam1 complex make it an excellent protein complex model to generally evaluate protein complex stabilization and separation using native CIEF.

Dam1 is a stable complex, but in order to confirm the association of the Dam1 complex at 15°C, we used increasing capillary temperature to monitor the dissociation of the complex. The results are illustrated in figure 3A. Comparison of the native CIEF electropherograms from increased separation temperatures appear to show a general trend of increased dissociation of the Dam1 complex to its components. From 20°C to 30°C, the large broad peak completely disappears and the small sharp peak intensities increase, indicative of thermal dissociation of the complex to its components. Thus, the largest broad peak, most prominent in the 15°C and 20°C electropherograms, can be indirectly identified as the intact Dam1 complex. Small sharp peaks in the separation at 15°C indicate a minor amount of dissociation of the Dam1 complex to its components.

The Dam1 complex peak is unusually broad for CIEF. This was likely due to aggregation since sample was overloaded (1.2 μg) to adequately detect the complex dissociation components. Aggregation of antibodies during CIEF yield similar results.⁵¹ To test the possibility that Dam1 dissociates upon focusing from capillary overloading, a separation of Dam1 under normal loading conditions (120 ng) was performed (Figure 4A). An extremely sharp peak with a few other small peaks was observed. These results indicate that either the Dam1 complex remains completely intact when not overloaded or that the dissociated protein signals are below the limit of detection.

Dam1 subcomplex separation

One might expect that as the separation temperature was increased, the Dam1 complex would lose individual proteins one at a time until it was fully dissociated into its 10 components (listed in table 1). With each increase in separation temperature an increasing number of peaks should have been detected until a maximum was reached. However, after close examination of the number of peaks from dissociation of the Dam1 complex at different temperatures it became obvious that the electropherogram from separations performed at 25°C, and not 30°C, had the most peaks. This is clearly illustrated in the viscosity corrected and expanded separations in figure 3B. This counterintuitive trend was likely due to the partial dissociation of the complex into subcomplexes with higher thermodynamic stability than the complex itself. Peaks present at 25°C, but not 30°C, were marked (•) as likely subcomplexes. At 30°C, the Dam1 complex and its subcomplexes appear to be completely dissociated into their component proteins which were then focused individually to their isoelectric points. Note there are fewer than 10 protein peaks in the 30°C separation. This was likely a detection issue since only 7 of the 10 Dam1 components have tryptophan (see table 1), the highest adsorbing residue at 280 nm. Tyrosine is present in all Dam1 complex components, but has a ~ 5-fold lower extinction coefficient.⁶⁵ Three of the peaks present at all separation temperatures, indicated by the black boxes spanning all separation temperatures, were categorized as either individual proteins which do not interact with the Dam1 complex under the given conditions, system peaks from separations under overloading conditions, or impurities. Four other component proteins are only detected individually at 25°C and 30°C, indicated by blue rectangles spanning only those temperatures. The red boxes designate subcomplexes that are present at temperatures lower than 25°C and thus bound to the Dam1 complex less tightly.

The direct observation of subcomplexes was not new and has been shown for the well-studied RNA polymerase III using traditional molecular biology techniques⁶⁶ and native mass spectrometry.²¹ Similarly, a bioinformatic analysis of four global protein complex datasets from yeast identified statistically probable subcomplexes.²⁴ However, by performing the native CIEF separations at different temperatures in solution, we will be able to understand and quantify the thermodynamic characteristics of protein subcomplexes within a given protein complex. A similar strategy was useful for native MS analysis of the solution-phase equilibria of a small heat shock protein.²²

Dam1 complex phosphorylation state separation

CIEF has proven useful for identification and quantification of protein phosphorylation due to the inherent isoelectric point shift associated with addition of a phosphate moiety to a serine, threonine, or tyrosine residue.^{67, 68} We were able to extend this analysis to protein complexes using native CIEF through detection of the isoelectric point shift of Dam1 phosphorylation in vitro by Mps1 kinase. The CIEF results are shown in figure 4. Addition of purified Mps1 kinase causes an average stoichiometry of phosphorylation of 8.3 moles phosphate per mole of Dam1 complex from a radioactivity kinase assay (unpublished results). The Mps1 kinase was 50-fold lower in concentration than the Dam1 complex and

thus below the limit of detection. Therefore, we reason that both sharp peaks in figure 4B are the Dam1 complex with different phosphorylation stoichiometries. Based on the peak height ratio (2:1), the close proximity of the peaks (similar pI and thus phosphorylation state), and the average stoichiometry of 8.3 from the radioactivity assay, we hypothesize that the two phosphorylation states of the Dam1 complex are either hepta- and nona-phosphorylated or penta- and deca-phosphorylated. However, further analysis with mass spectrometry will be necessary to elucidate the phosphorylation stoichiometry of the two states. Analysis of non-stoichiometric phosphorylation of proteins is a constant challenge using mass spectrometry.⁶⁹ The ability to differentiate and quantify different phosphorylation stoichiometries, particularly of protein complexes, their isoforms, and subcomplexes, will be highly beneficial in numerous biological studies.

The peak height ratio of the differentially phosphorylated Dam1 complex was used to estimate their phosphorylation states, yet there is a discrepancy in the peak heights of the phosphorylated and unphosphorylated Dam1 complexes (Figure 4A versus 4B). We believe this may have been due to loss of the unphosphorylated Dam1 complex during focusing due to EOF. For these separations, we used in-house hydroxypropyl cellulose-coated capillaries which may have inactivated the capillary surface to different degrees. Since the unphosphorylated Dam1 complex is nearer to the capillary outlet, a fraction of it could have been mobilized out of the capillary during the focusing step prior to detection. The slower focusing rate of the unphosphorylated Dam1 complex, illustrated in the 0–10 min region of figures 4A and 4B, further supports this idea. Additionally, this explains the much shorter mobilization times for the Dam1 complex in figure 4 relative to figure 3. Thus, the peak heights of the phosphorylated Dam1 complex should be more accurate and comparable than the unphosphorylated Dam1 complex peak height.

mTORC1 and 2 protein complex isoform separation

The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase known to be involved in cell metabolism, growth, and survival. It has been implicated in cancer, type 2 diabetes, and neurodegenerative diseases.⁷⁰ mTOR participates in two distinct complexes, mTORC1 and mTORC2, making it an ideal model protein complex isoform. The rapamycin-sensitive mTOR signaling complex (mTORC1), composed of mTOR, Raptor, mLST8, PRAS40, and Deptor, regulates cell growth and translation through phosphorylation of eIF-4E binding protein 1 and S6 kinase.^{71–75} The rapamycin-insensitive mTOR signaling complex (mTORC2), composed of mTOR, Rictor, mLST8, mSIN1, Protor, and Deptor, controls cell proliferation and survival by phosphorylating and activating the Akt/PKB kinase.^{75–81} A method for separating mTORC1 and 2 for further biochemical characterization would improve our understanding of the function of each complex and its role in human diseases. Since both complexes exist in the same cellular environment⁸² and are theoretically similar in size and isoelectric point (Table 3), their separation is a formidable challenge. Previous efforts to separate mTORC1 and 2 using conventional gel filtration chromatography had neither the resolution nor the short analysis time to maintain mTORC2 complex integrity.⁸³

Using native CIEF we were able to resolve mTORC1 and 2, shown in figure 5A. As expected, due to the similar components of the complexes the high efficiency CIEF separation technique was necessary to resolve the complex isoforms with very similar isoelectric points. Using isoelectric point markers, we estimate the isoelectric points of the complex isoforms are 5.88 (mTORC1) and 5.94 (mTORC2). Comparison of the peak areas for the two complexes in figure 5A indicates that, under the conditions for isolation and separation, there is double the amount of mTORC1 present relative to mTORC2. Addition of a narrow range of ampholytes (pH 4 – 6.5) was necessary to achieve resolution and possibly maintain mTORC2 stability, illustrated by comparison of figure 5A and 5D. We

used the narrow range ampholytes to decrease the slope of the pH gradient in the pH 4 – 6.5 region and to increase resolution of the already established pH 3–10 gradient. CIEF separations have been performed with only small range ampholytes,⁵¹ but the combination of ampholytes allows for monitoring a larger pH range while improving resolution in a region of interest.⁸⁴

The native conditions and short analysis time of CIEF made separation and maintenance of the unstable mTORC2 possible. Upon replicate analysis after storage with carrier ampholytes at 4°C for five hours, the mTORC2 peak completely disappeared as seen in figure 5B. Similar results were found from CIEF separations of protein complexes from *E. coli* extracts stored in ampholytes for extended periods.⁴⁷ mTORC1 was more stable and was still detectable in replicate runs well after 5 hours in carrier ampholytes at 4°C (data not shown). From electron microscopy experiments, the complexes were expected to have lifetimes of ca. four days at 4°C in purification buffer.⁸³ Indeed, we were able to observe nearly complete dissociation of the complexes after seven days at 4°C shown in figure 5C. Thus, native CIEF may also be a cheap, fast, and automated method for characterization of protein complex stability.

CONCLUSIONS

We have demonstrated that native CIEF is a powerful technique for separating large protein complex isoforms and subcomplexes. The capability to resolve these complexes in solution instead of in the gas phase, as in native mass spectrometry, presents the possibility of performing numerous targeted analyses of protein complex composition, post-translational modification state, architecture, stability, equilibrium, and relative abundance under biologically relevant conditions. Further improvements to the CIEF instrumentation to lower separation temperatures and stabilize weakly associated protein complexes like mTORC2 will benefit the aforementioned experiments and allow for accurate quantification of protein complex isoform ratios. Utilization of a more sensitive and selective detector, such as native laser-induced fluorescence or mass spectrometry, would also expand the capabilities of native CIEF. In these experiments, proteins from the dissociated Dam1 complex were detected only when overloaded and mTORC1 and 2 component proteins were barely above the limit of detection at the purification concentration analyzed. Additionally, the coupling of native CIEF to a mass spectrometer would strengthen both native CIEF and native mass spectrometry. Most native mass spectrometry experiments have been performed on highly-abundant, easily-purified, stable complexes such as RNA polymerase III,²¹ the 20S proteasome,¹⁵ and GroEL.¹⁶ Recent advances in stabilization of soluble^{17, 55} and membrane bound^{56, 57} protein complexes will surely expand the capabilities for analyzing biologically-interesting, uncharacterized complexes. However, native CIEF could drastically simplify the need for structural maintenance of protein complexes during the electrospray and ion transmission processes; resolution of protein complex isoforms and subcomplexes could be achieved prior to mass spectrometric detection. Native CIEF could also expand the capabilities of native mass spectrometry from the added purification of protein complexes prior to the electrospray process.

Biochemical interactions are known to be dependent on pH.²⁶ Thus, it is possible that native CIEF separation may not be compatible with protein complexes that are sensitive to pH changes. To our knowledge, there have not been any studies which investigate the effect of the exposure of biochemical interactions to different pHs during CIEF. The point detector used for these CIEF experiments did not allow monitoring of complexes during the separation process. However, the stability of these interactions at different pHs could be probed using CE with different pH buffers or CIEF with whole-column detection.

Additionally, analysis of other purified native complexes with different biochemical properties will shed further light on this phenomenon.

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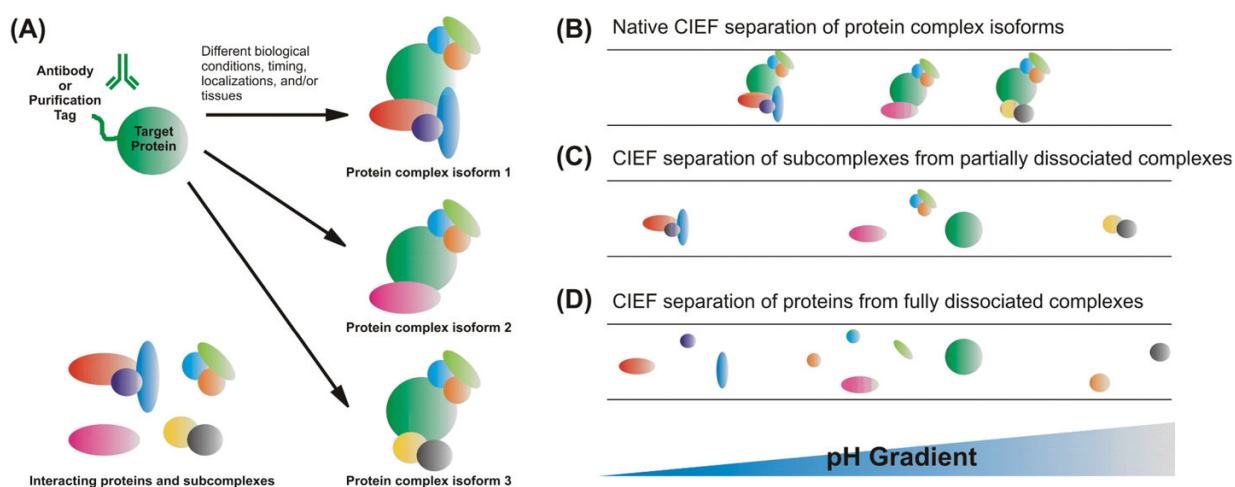


Figure 1.

Schematic of protein complex isoforms and subcomplexes and their CIEF separations. (A) The composition of protein complexes are often elucidated through purification of a target protein, and all proteins associated with it, using an antibody or purification tag. The purification creates a heterogeneous mixture of protein complexes, called protein complex isoforms. Three examples are shown here. Each protein complex isoform can be composed of different proteins or subcomplexes depending on its specific function under certain biological condition. (B) A theoretical native CIEF separation of the three protein complex isoforms. Although many proteins are similar in each protein complex isoform, the proteins or subcomplexes which are different shift the isoelectric point of the protein complex isoform. This inherent pI shift allows for separation within the pH gradient generated using CIEF. (C) Dissociated protein complexes are known to maintain more thermodynamically stable subcomplexes. These subcomplexes can also be separated based on their isoelectric points using native CIEF. (D) A traditional CIEF separation of individual proteins from fully dissociated protein complexes isoforms and subcomplexes using denaturants or thermal dissociation. Note that the pIs of the separated proteins contribute, likely as a weighted average, to the pIs of the subcomplexes they are associated with in the separation above. Likewise, comparison of the subcomplex separation in (B) to the complex isoform separation in (A) illustrates how the pIs of proteins and subcomplexes contribute to the pI of the complex isoform.

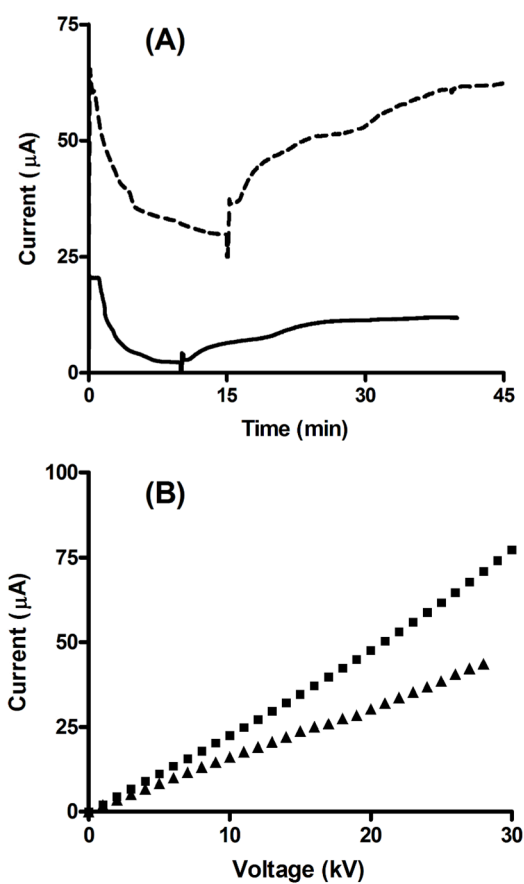


Figure 2. (A) CIEF current traces for 50 μm (—) and 100 μm (- -) ID capillaries. The current trace for the 100 μm ID capillary was offset 25 μA for clarity. (B) Ω plot for the focusing (■) and mobilization (▲) steps using a 100 μm ID capillary.

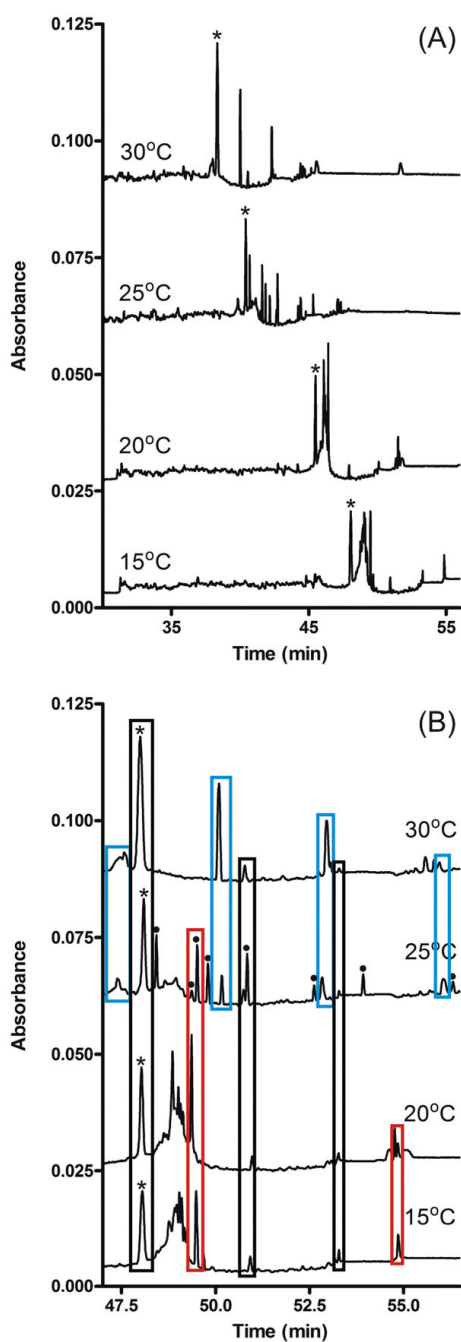


Figure 3.

Thermal dissociation studies of the Dam1 complex. (A) CIEF separations of 500 $\mu\text{g/mL}$ Dam1 complex at different separation temperatures using a 100 μm HPC coated capillary. The peaks marked with (*) were used as a mobilization marker for viscosity corrections. (B) The same CIEF separations from (A) viscosity corrected and expanded in the region of Dam1 complex peaks. Potential subcomplex peaks were marked with (●) in the 25°C separation. Peaks that were common to specific separation temperatures were enclosed with a box, marking individual proteins (blue), subcomplexes (red), and potentially individual proteins, system peaks, or impurities (black).

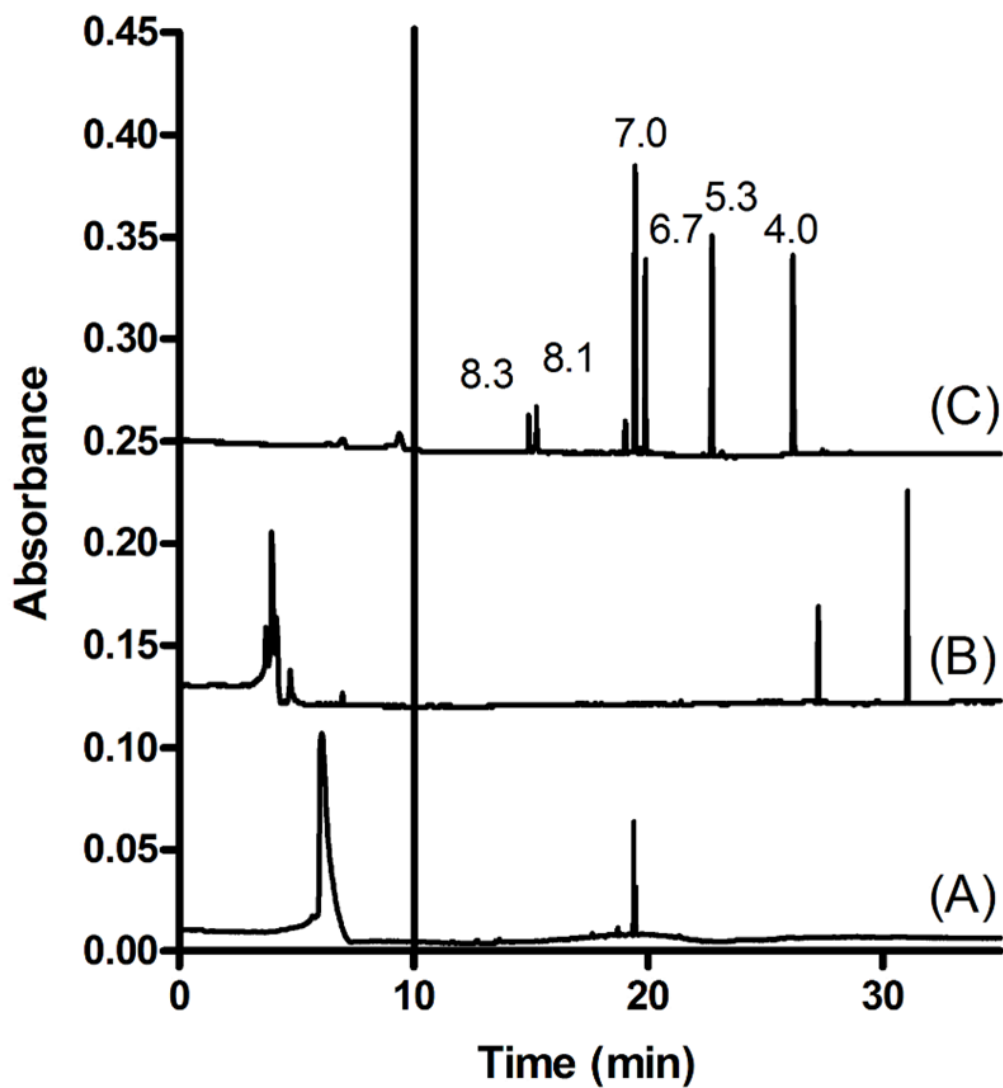


Figure 4. Dam1 complex phosphorylation analysis. CIEF separations of (A) 50 $\mu\text{g/mL}$ purified Dam1 complex, (B) 50 $\mu\text{g/mL}$ Dam1 with $\sim 1\mu\text{g/mL}$ Mps1 kinase, and (C) 10 μM pI markers for monitoring pI shift in Dam1 using a 100 μm HPC coated capillary. The focusing and mobilization steps were differentiated using a solid vertical line at 10 min.

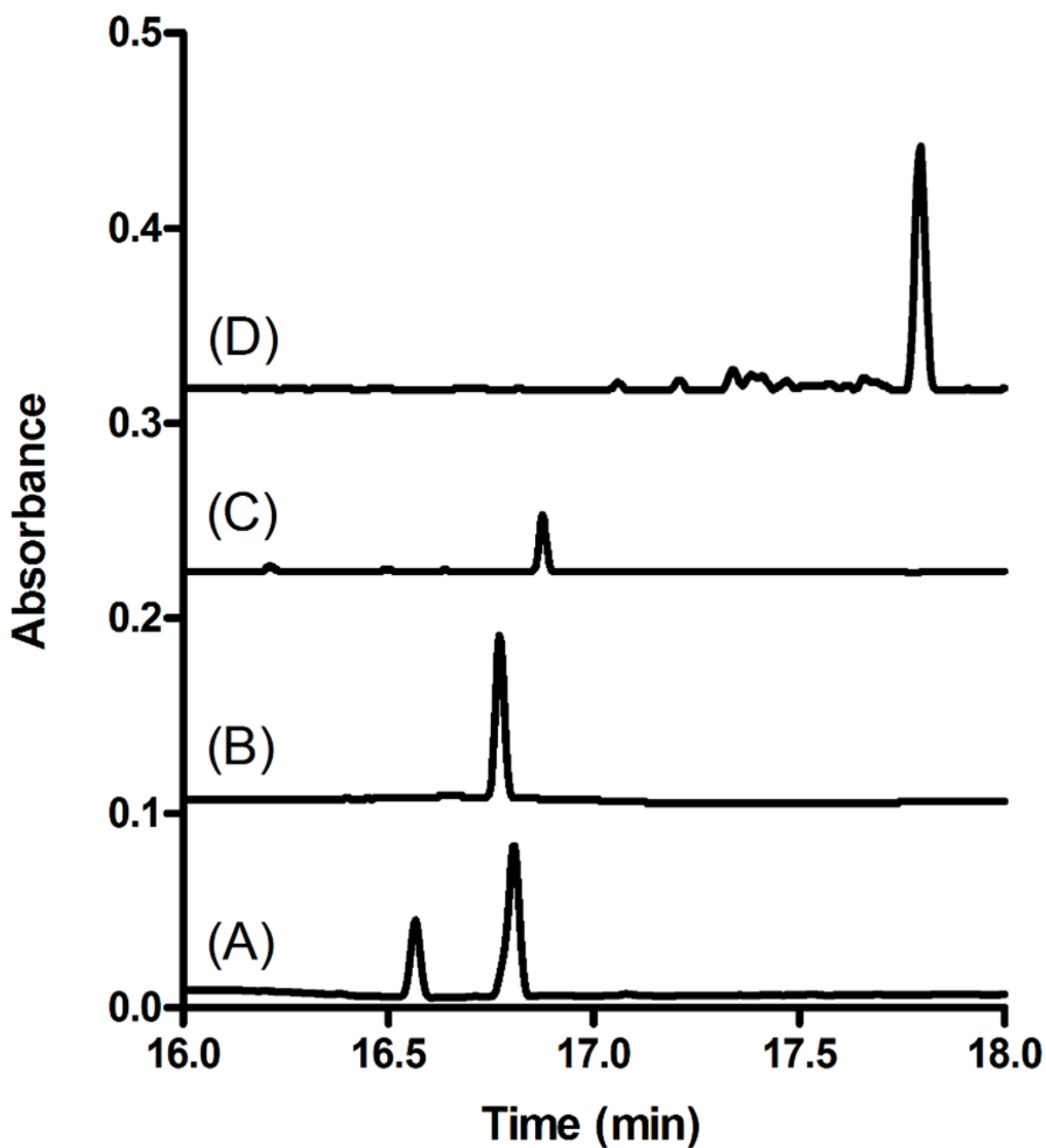


Figure 5. CIEF separations of 25 μ g/mL mTORC1 and 2 with different conditions using a 50 μ m PVA coated capillary. (A) Successful separation of mTORC1 and 2 upon immediate analysis with 1.7% w/v pI 3 – 10 and 2.1% w/v pI 4 – 6.5 ampholytes. (B) Replicate analysis of mTORC1 and 2 as in (A) after 5 hours. (C) Separation of dissociated mTORC1 and 2 as in (A) after storage at 4 $^{\circ}$ C for one week. (D) Unsuccessful separation of mTORC1 and 2 using only 1.7% w/v pI 3 – 10 ampholytes.

Table 1

The known, confirmed protein components making up the Dam1 complex with their theoretical molecular weights and isoelectric points, calculated using ExPASy (www.expasy.org) with sequences from the *Saccharomyces* Genome Database (<http://www.yeastgenome.org/>). Note that post-translational modifications are not accounted for in the calculations. The number of tryptophan (W) and tyrosine (Y) residues, necessary for UV detection at 280 nm, were counted from their protein sequences.

Protein	MW (kDa)	pI	No. of W's	No. of Y's
Dam1	38.4	9.2	1	6
Spc34-6XHis	34.9	8.3	1	9
Duo1	27.5	10.0	2	4
Dad1	10.5	4.2	2	13
Dad2	15.1	4.1	2	2
Dad3	10.8	5.3	-	3
Dad4	8.2	6.7	-	1
Spc19	18.9	4.8	-	3
Ask1	32.1	4.6	1	2
Hsk3	8.1	7.9	1	4
Total	204.5		10	47
Average		6.5	1	4.7

Table 2

List of viscosity correction (ν) values based on either migration time (t) or average mobilization current (I). Migration time corrections in figure 3B were made using ν_t values.

Separation temperature (°C)	ν_t	ν_I
15	1	1
20	1.06	1.07
25	1.19	1.21
30	1.25	1.31

Table 3

The known, confirmed protein components making up mTORC1 and 2 with their theoretical molecular weights and isoelectric points, calculated using ExPASy (www.expasy.org) from human protein sequences listed by NIH (www.ncbi.nlm.nih.gov/protein). Note that the theoretical molecular weight and pI may be inaccurate since some unknown proteins may have yet to be identified in the complexes and post-translational modifications are not accounted for in the calculations.

mTORC1	MW (kDa)	pI	mTORC2	MW (kDa)	pI
mTOR	288.9	6.7	mTOR	288.9	6.7
mLST8	35.9	5.5	mLST8	35.9	5.5
Deptor	46.3	8.3	Deptor	46.3	8.3
Raptor	149.0	6.4	Rictor	192.2	7.2
PRAS40	27.4	4.7	Protor	40.9	6.3
			SINI	59.1	7.2
Total	547.5		Total	665.1	
Average		6.3	Average		7.2