EuPA OPEN PROTEOMICS 7 (2015) 64–72

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: http://www.elsevier.com/locate/euprot

Review

Deciphering metabolic networks by blue native polyacrylamide gel electrophoresis: A functional proteomic exploration

*Christopher Auger, Nishma D. Appanna, Azhar Alhasawi, Vasu D. Appanna**

Faculty of Science and Engineering Laurentian University, Sudbury, Ontario P3E 2C6, Canada

ARTICLE INFO

Article history:
Received 16 March 2015
Received in revised form
11 May 2015
Accepted 18 May 2015
Available online 27 May 2015

Keywords:
Enzymes
Electrophoresis
Protein–protein interactions
Metabolism
Enzymology
Proteomics

ABSTRACT

Metabolism is the consortium of reactions within a cell which directs a variety of processes including energy synthesis, signalling and the behaviour of a biological system. Metabolic networks, and more specifically the activity of enzymes within them, provide an accurate status of how cellular information is being executed. The performance of these networks and their ability to siphon metabolites in a number of directions may be the difference between a healthy and diseased state. Blue native polyacrylamide gel electrophoresis (BN-PAGE), owing to its simplicity and wide-ranging applications, permits the inspection of these nodules. The separation of proteins and enzyme complexes in their native format enables the exploration of enzymatic activity in metabolic networks via in-gel assays. These are quick, specific, and amenable to further studies. This electrophoretic technology not only enables the visualization of enzymatic efficacy but reveals the crosstalk among enzymes and their interactions with other organellar partners.

Contents

* Corresponding author. Tel.: +1 705 675 1151x2112; fax: +1 705 675 4844.
E-mail address: vappanna@laurentian.ca (V.D. Appanna).

http://dx.doi.org/10.1016/j.euprot.2015.05.003

1. Introduction

Metabolism is the foundation of all living organisms as it fulfils a multitude of crucial functions that dictate their survival, proliferation and in essence, existence. It is broadly divided into catabolism and anabolism. The latter involves the genesis of biomolecules and the former implicates the degradation of substrates [1]. These constitute the inner workings of an organism and are characterized by an array of metabolic networks that guides most cellular processes. These networks are orchestrated by enzymes that conjugate their efforts to bring a series of biochemical reactions to fruition [2,3]. For instance, glycolysis is dedicated to the catabolism of D-glucose with the effect of generating essential metabolites like ATP and pyruvate [4]. Depending on the needs of the cell, the latter may be shuttled to the tricarboxylic acid (TCA) cycle, a metabolic module which extracts reducing factors such as NADH and $FADH_2$ [5]. These enzymes often work in close association and are usually in close proximity to allow for the effective channelling of substrates and for proper regulatory control [6]. Therefore, probing such multi-enzymatic complexes requires non-invasive tools that will ensure the preservation of their activity [7].

Polyacrylamide gel electrophoresis is a technique that lends itself to the study of biomolecules in a wide range of fields, from biotechnology to molecular biology and forensic science [8]. Its ability to separate moieties such as proteins, lipids, and nucleic acids in native forms grants investigators the capacity to inspect these molecular components [9]. Indeed, great leaps in molecular medicine would not have been possible without this critical tool. By excluding strong detergents (e.g., sodium dodecyl sulfate) and utilizing Coomassie blue G-250 to induce a charge shift in proteins, blue native polyacrylamide gel electrophoresis (BN-PAGE) was originally developed as a means of separating membrane complexes in enzymatically active form [10]. However, modifications to this electrophoretic technique and the emergence of novel in-gel activity assays have allowed researchers to analyze a wider spectrum of enzymes, membrane proteins and many supra-complexes residing in biological compartments [11–13]. The modulation of acrylamide concentration and introduction of micelle-forming chemicals have extended the analytical flexibility of this technique [13,14]. The fine-tuning of this electrophoretic technique has permitted the investigation of key enzymatic activities in metabolic networks and has yielded precise information on complex cellular processes [8,15].

Although these entities may be probed individually, BN-PAGE provides a relatively non-invasive technique to explore how these biomolecules function collaboratively. The ability of this analytical tool to maintain proteins and their superstructures in native conditions enables the molecular visualization of these enzymes in a relatively undisturbed fashion [16]. This procedure also tends to have minimal impact on the transient forces that orchestrate diverse enzymes to congregate for their select metabolic tasks. Hence, the exploration of these complexes and some of the key enzymes that dictate the occurrence of a specific metabolic network generate an accurate functional landscape of a particular metabolic nodule and its performance at a given time [17].

2. Energy-generating networks

As the primary generator of the universal energy currency adenosine triphosphate (ATP) in aerobic organisms, the status of oxidative phosphorylation and the electron transport chain (ETC) complexes provide a strong gauge of energetic metabolism and mitochondrial well-being [18]. NADH and $FADH_2$ provide the reducing power necessary to generate a proton gradient in complexes I–IV across the internal mitochondrial membrane. In turn, complex V harnesses this gradient to catalyze the formation of the high-energy compound ATP from adenosine diphosphate (ADP) [19]. The need to study the activity of ETC components arose from the identification of a number of neuromuscular disorders stemming from faulty mitochondrial activity. Genetic mutations in the oxidative phosphorylation complexes affect 1 in 5000 individuals, and would be troublesome to characterize without specialized enzyme assays [20,21]. In addition, environmental factors, such as metal stress, reactive oxygen and nitrogen species (ROS and RNS, respectively) are capable of disrupting this essential ATP-making machinery [22,23]. The inherent difficulty in studying ETC proteins lies in their hydrophobicity and highly complex structures. A strong detergent such as SDS leads to the dissociation of these multi-subunit structures, rendering activity assays ineffective [24].

To counter this, Schägger and von Jagow employed the mild detergent *n*-dodecyl β-D-maltoside (DDM), thus rendering the complexes soluble for electrophoresis and maintaining their superstructure [10]. Variations to the standard protocol, such as the substitution of digitonin for DDM, as well as the development of clear native PAGE, has led to the identification of all the ETC complexes [13]. Once separated, the activity of this ATP-generating machinery can be elucidated via specific in-gel enzymatic activity assays [11]. The formation of reducing cofactors like NADH, NADPH and $FADH_2$ can be readily visualized by the generation of formazan at the site of enzymatic activity in the presence of phenazine methosulfate (PMS) and iodonitrotetrazolium (INT) salts [25]. The substitution of PMS with 2,6-dichlorophenolindophenol (DCPIP) permits the monitoring of the enzymatic oxidation of reduced cofactors [26,27]. In some cases, products which are not amenable to redox processes can be coupled with redox enzymes in order to reveal their localization in the gel (Fig. 1) [26–28].

For instance, complexes I (EC 1.6.5.3) and II (EC 1.3.5.1) can be detected via the addition of their preferred substrates (NADH and succinic acid, respectively) and an indicator such as PMS coupled to INT [11,14]. The latter, when reduced, produces a pink-coloured formazan precipitate at the location of the complex in-gel. A reaction mixture consisting of cytochrome C and the indicator 3.3′-diaminobenzidine is used to pinpoint complex IV (EC 1.9.3.1), while ADP and $Pb(NO_3)_2$ are used to observe complex V (EC 3.6.3.14) [11,14,29]. These activity assays permit the qualitative comparison of ETC activity between disparate conditions, such as a healthy and diseased state. While semi-quantitative measurements are feasible using densitometric software such as ImageJ, precise

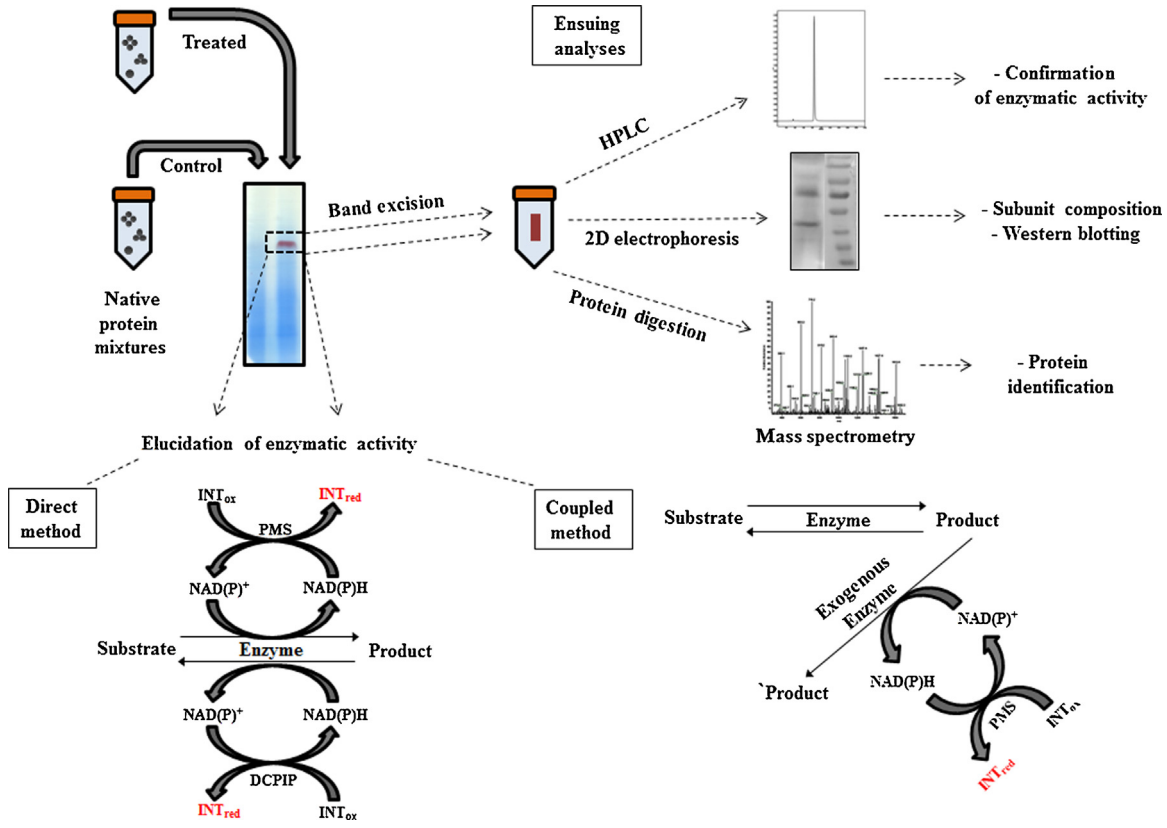

Fig. 1 – BN-PAGE and secondary analyses. Following separation of the native protein mixture via electrophoresis, enzymatic activity can be elucidated by specific direct or enzyme-coupled in-gel activity assays. The activity bands are amenable to complementary assays which ascertain the nature of the protein [10,13,23]. INT, iodonitrotetrazolium; PMS, phenazine methosulfate.

determination of enzymatic activity necessitates other analytical assays [28]. Although the maintenance of complex structure and activity is valuable, another benefit of BN-PAGE is the ability to uncover novel functional interactions between mitochondrial proteins. For instance, there appears to be a weak association between complex I and creatine kinase, as evidenced by the latter migrating at a higher molecular mass than the enzyme would if unaccompanied [30]. This technique also identified the association of cytochrome C with complexes III and IV of the ETC [30]. Further analysis, such as band excision followed by two-dimensional SDS-PAGE, Western blotting and mass spectrometry enables detailed characterizations of these proteins (Fig. 1) [23,30–32].

While the primary role of the TCA cycle is to provide the reducing factors necessary to power oxidative phosphorylation and the generation of ATP, the malleability of this metabolic hub is often overlooked [33]. If energy is not required, or the ETC is defective due to inherent or environmental factors, citric acid may be diverted towards gluconeogenesis, or the production of ATP via substrate-level phosphorylation [22,34]. Key enzymes in the TCA cycle can be observed using BN-PAGE in order to gauge the status of this network. Given the susceptibility of iron (Fe) – sulfur (S) cluster-containing proteins like aconitase (ACN, EC 4.2.1.3) to succumb to the dangers of an oxidative environment, tricarballylic acid is added to stabilize the enzyme during electrophoresis. ACN activity is uncovered by coupling the formation of its product isocitrate at the site of the enzyme to exogenous isocitrate dehydrogenase (ICDH), NAD, PMS and INT [25]. The activity of NAD^+-dependent ICDH (ICDH-NAD, EC 1.1.1.41) can also be measured using a reaction mixture consisting of its substrates (isocitrate, NAD^+), PMS and INT [35]. Isoenzymes can be visualized as distinct activity bands in the gel [25]. The α-ketoglutarate dehydrogenase (KGDH, EC 1.2.4.2) and pyruvate dehydrogenase (PDH, EC 1.2.4.1) complexes are sensitive to the presence of peroxide and peroxynitrite due to the presence of a lipoic acid residue in the active site [36,37]. KGDH and PDH dysfunctions have readily been revealed by BN-PAGE. However, the large molecular mass of PDH necessitates a native agarose gel in lieu of acrylamide to separate this protein [38–40].

Fumarase (FUM EC 4.2.1.2), which catalyzes the reversible production of malate from fumarate, can be assessed by coupling the production of the former to exogenously added malate dehydrogenase (MDH) and INT [26]. The oxidation of malate to oxaloacetate, the final step of the TCA cycle, is catalyzed by MDH (EC 1.1.1.37) and is assessed using a reaction mixture consisting of malate, NAD^+, INT and PMS [35]. It is not uncommon for enzymes to congregate to channel metabolites from one enzyme to another; a molecular arrangement referred to as a metabolon [41,42]. However, these transient associations are generally sensitive to experimental manipulation [7]. BN-PAGE has enabled the identification of a complex

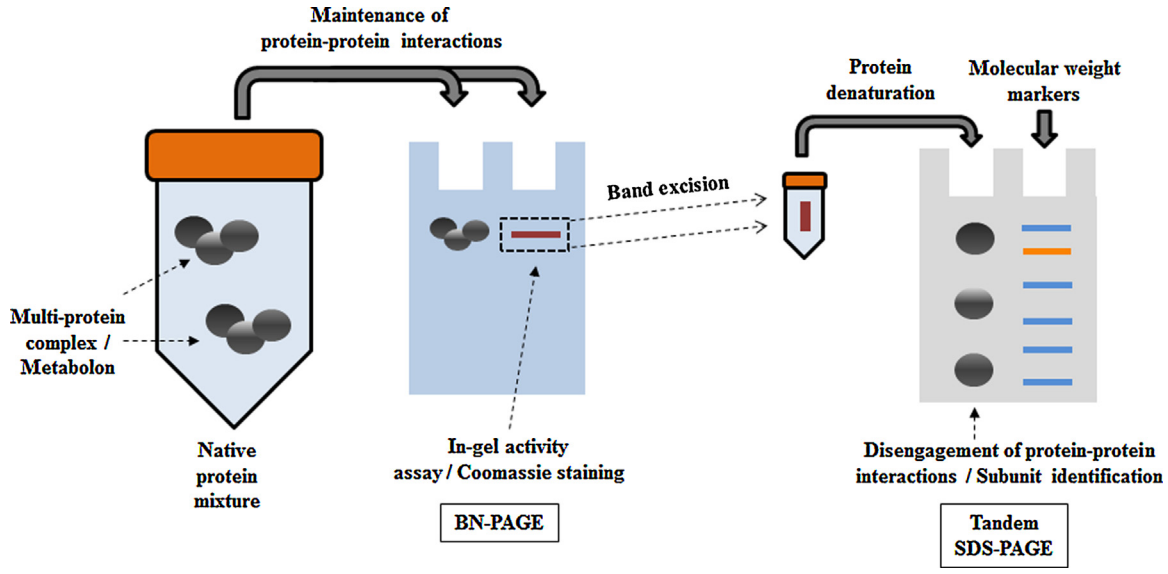

Fig. 2 – BN-PAGE as a tool to uncover multi-protein complexes. Protein–protein interactions maintained during BN-PAGE can be disrupted and analyzed following tandem SDS-PAGE. In-gel activity assays can be applied to identify the localization of metabolons, complexes consisting of two or more intimately linked enzymes [23,26,28].

consisting of MDH and citrate synthase in human embryonic stem cells [31]. This analytical tool not only provides an actual snapshot of the oxidative ATP machinery but also reveals how these enzymes conjugate in an effort to ensure the efficiency of the process (Fig. 2).

Although NADH and $FADH_2$ help generate ATP via oxidative phosphorylation, this process tends to create an oxidative environment that has to be controlled [43–45]. BN-PAGE is a powerful tool to monitor the redox status of a cell by tracking the activity of NADPH-producing enzymes [46,47]. This reductive cofactor not only orchestrates the homeostasis of oxidative tension but also participates in biosynthetic reactions [48–50]. Living systems primarily employ $NADP^+$-dependent ICDH (ICDH-NADP, EC 1.1.1.42), malic enzyme (ME, EC 1.1.1.40) and G6PDH (EC 1.1.1.49) for the synthesis of NADPH. These three soluble NADPH-generating proteins can be tracked following BN-PAGE using reaction mixtures containing NADP, INT and PMS [47]. ICDH-NADP is analyzed via the same protocol as ICDH-NAD, with $NADP^+$ substituting for NAD^+ in the reaction [51]. ME, which catalyzes the formation of pyruvate and NADPH from malate, requires the latter and $NADP^+$ to stimulate its activity [52]. G6PDH is probed in a similar manner, but with glucose-6-phosphate included in place of malate. Interestingly, it's possible to investigate the presence of multiple isoforms of G6PDH by in-gel activity assays [53]. NAD kinase (EC 2.7.1.23), an enzyme which phosphorylates NAD^+ to generate the cofactor $NADP^+$, is pivotal for redox balance [54,55]. It can be made apparent using a reaction mixture consisting of NAD^+, ATP, ICDH-NADP, PMS and INT [56].

3. D-Glucose and fatty acid metabolism

The interaction of soluble enzymes with the Coomassie G-250 dye, and the utilization of gradient gels render enzymes involved in glycolysis and lipogenesis amenable to BN-PAGE [40,57]. Hexokinase (HK, EC 2.7.1.1), glucose-6-phosphate isomerase (G6PI, EC 5.3.1.9), pyruvate kinase (PK, EC 2.7.1.40) and lactate dehydrogenase (LDH, EC 1.1.1.27) can be assessed [23,28,29,58,59]. The detection of HK and G6PI proceeds via a coupling reaction, whereby the substrates (glucose and ATP for HK; fructose-6-phosphate for G6PI) are included in the reaction mixture, and enzymatic activity is elucidated by linking the formation of glucose-6-phopshate to exogenously added glucose-6-phosphate dehydrogenase (G6PDH) with $NADP^+$, INT and PMS [29]. PK is visualized with a reaction mixture consisting of phosphoenolpyruvate (PEP), ADP, LDH, NADH, 2,6-dichlorophenolindophenol (DCPIP) and INT23. Interestingly, substituting AMP and inorganic phosphate (P_i) for ADP allows for the detection of PEP synthase (PEPS, EC 2.7.9.2), a reversible PEP-dependent kinase which plays a role in CO_2 fixation [28,60]. By substituting P_i with inorganic pyrophosphate (PP_i), it is also possible to spot the activity of pyruvate orthophosphate dikinase (PPDK, EC 2.7.9.1), a key energy-generator in such infectious parasites as *Entamoeba histolytica* [28,61,62].

LDH, which plays a crucial role in anaerobic metabolism and fermentation, is elucidated with the aid of lactate, NAD, INT and PMS [40,63]. Indeed, such analyses have helped unearth the diverse roles of this enzyme in the mitochondria and nucleus [40,58,59]. To further validate the ability of this technique to maintain low-affinity protein–protein interactions, BN-PAGE retardation assays can be performed to determine whether such associations exist. This method has been applied to demonstrate a physical link between triosephosphate isomerase (TPI) and dextran, a microbial polysaccharide [64]. A superstructure composed of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), fructose-bisphosphate aldolase, phosphoglycerate kinase 1 and TPI was recently identified by BN-PAGE followed

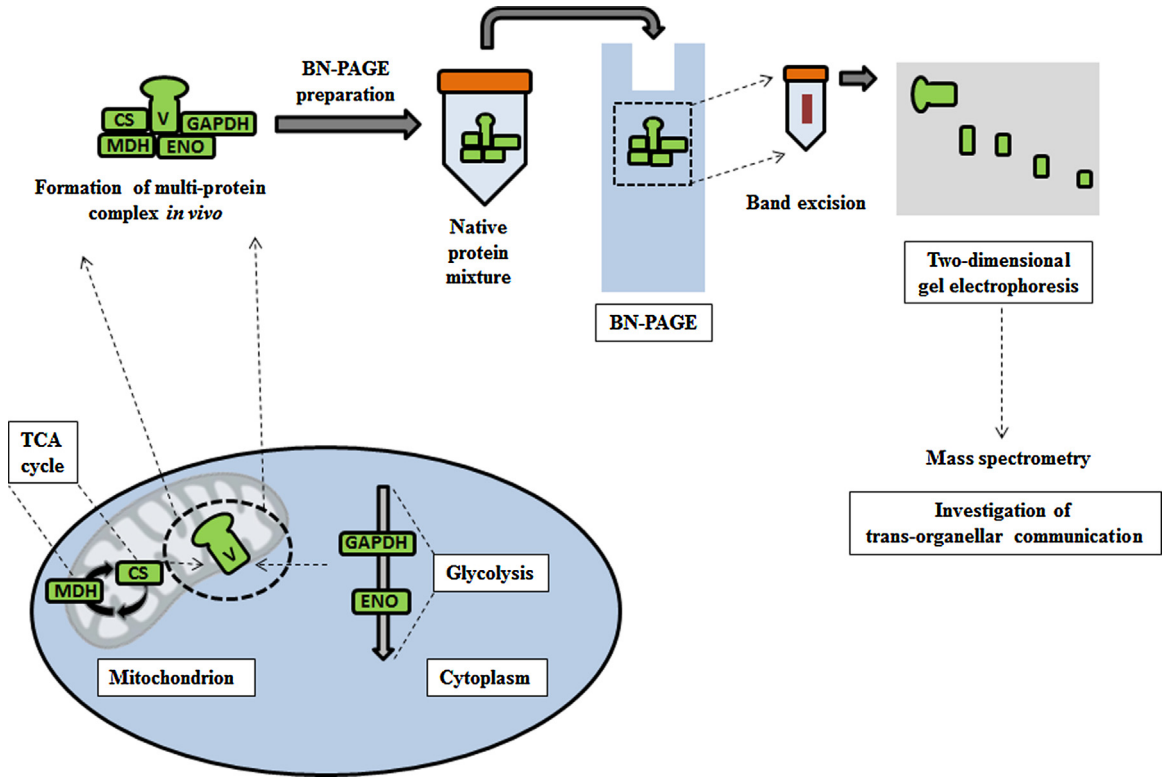

Fig. 3 – Trans-organellar complexes identified by BN-PAGE. The maintenance of protein–protein interactions allows the detection of multi-protein complexes consisting of enzymes from disparate compartments. Here, a complex consisting of enolase (ENO), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), citrate synthase (CS), malate dehydrogenase (MDH) and ATP synthetase (V) is investigated by BN-PAGE and secondary analyses [81,87].

by MALDI-TOF/TOF MS, indicating that these enzymes are likely to conjugate in vivo to carry out metabolic tasks (Fig. 3) [31].

The regeneration of glucose, a phenomenon important during fasting and starvation, is a ubiquitous process in all organisms. Enzymes crucial to this operation include pyruvate carboxylase (PC, EC 6.4.1.1). PEP carboxykinase (PEPCK, EC 4.1.1.32), and in some organisms, PEP carboxylase (PEPC, EC 4.1.1.31) [65–67]. While the latter is generally involved in CO_2 fixation in plants, PEPCK is crucial to the formation of PEP during gluconeogenesis. Both enzymes can be probed by BN-PAGE with the aid of the substrates; PEP, GDP/ADP and HCO_3^-/PEP and HCO_3^- for PEPCK and PEPC, respectively. The formation of oxaloacetate is coupled to MDH, NADH, DCPIP and INT [23,26]. PC, which in tandem with PEPCK can reverse the action of PK, can also be analyzed by linking oxaloacetate formation to MDH as described above. The necessary substrates; pyruvate, HCO_3^- and ATP, are included in the reaction mixture [26]. In addition, isocitrate lyase (ICL, EC 4.1.3.1), which allows plants, bacteria and fungi to bypass the decarboxylation that occurs in the TCA cycle, can be probed by in-gel activity assays [25]. When carbohydrates are required (e.g., for the synthesis of cell walls), lipids are broken down via ICL and subsequently channelled towards gluconeogenesis [68]. The analysis of ICL proceeds via a reaction mixture consisting of isocitrate, NAD, LDH, PMS and INT [69].

The activity of pivotal enzymes like acetyl-CoA carboxylase (ACC, EC 6.4.1.2), acetyl CoA synthetase (ACS, EC 6.2.1.1) and ATP-citrate lyase (EC 2.3.3.8) that mediate lipogenesis can be tracked by BN-PAGE [70,71]. Following electrophoresis, the gel is incubated in a reaction mixture with ATP, HCO_3^- and acetyl-CoA to detect ACC. The activity band is made visible using phosphate precipitation reagent [71]. The formation of acetyl-CoA by acetate is probed with a solution containing acetyl-CoA, AMP and PP_i. The generation of ATP is tracked using hexokinase, glucose, G6PDH and NADP, and the enzyme is spotted in-gel with PMS and INT [40]. The production of oxaloacetate mediated by ATP-citrate lyase is tracked with MDH, NADH, DCPIP and INT [71]. The breakdown of fatty acids by beta-oxidation is facilitated by L-carnitine, a moiety that transports lipids into the mitochondrial matrix for their subsequent degradation [72]. Two key enzymes involved in carnitine biosynthesis, namely γ-butyrobetainealdehyde dehydrogenase (BADH, EC 1.2.1.47) and butyrobetaine dioxygenase (BBDOX, EC 1.14.11.1) can be probed to study the formation of this non-essential amino acid [73]. BADH activity is monitored by adding γ-butyrobetaine, NADH, DCPIP, and INT in equilibration buffer. BBDOX activity is ascertained by the addition of γ-butyrobetaine, KG, sodium ascorbate, ferrous sulfate, and INT [73]. BN-PAGE was instrumental in detecting a PK and ATP-citrate synthase complex, the rate limiting step in the conversion of glucose to fatty acids in stem cells [31]. The ability of this method to capture transient metabolic links between pathways such as these is of the upmost importance as we attempt to unearth the dynamic workings of metabolic processes [74].

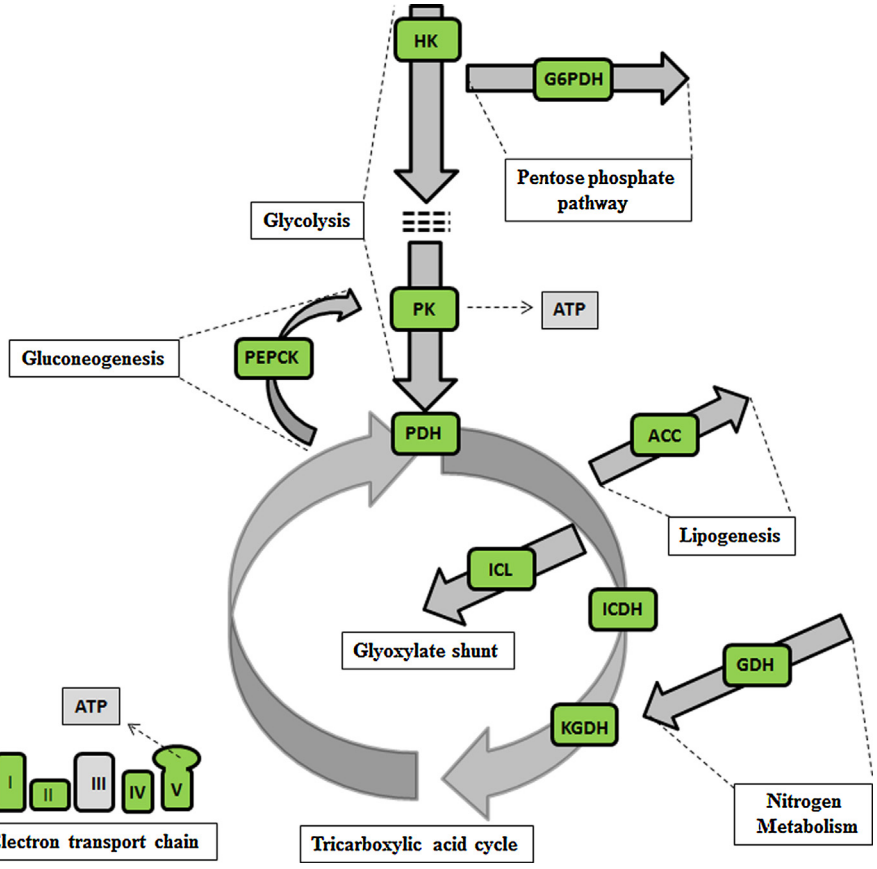

Fig. 4 – Metabolic enzymes investigated by BN-PAGE. Rate-limiting enzymes and those governing key intersections that have been analyzed by in-gel activity assays are highlighted in green [28,47,51,53,78]. I–V: Complexes of the electron transport chain; ACC, acetyl-CoA carboxylase; G6PDH, glucose-6-phosphate dehydrogenase; GDH, glutamate dehydrogenase; HK, hexokinase; ICDH, isocitrate dehydrogenase; ICL, isocitrate lyase; KGDH, alpha-ketoglutarate dehydrogenase; PDH, pyruvate dehydrogenase; PEPCK, phosphoenolpyruvate carboxykinase; PK, pyruvate kinase.

4. Nitrogen homeostasis

The maintenance and regulation of nitrogen metabolism is necessary for the proliferation and survival of organisms. Glutamate and glutamine are two metabolites that play key roles in the homeostasis of nitrogen, a process mediated by such enzymes as glutamate dehydrogenase (GDH) and glutaminase (GS) (Fig. 4) [75]. Both NAD^+ and $NADP^+$ dependent forms of GDH (EC 1.4.1.2 and EC 1.4.1.4) can be analyzed in-gel via the inclusion of glutamate, $NAD(P)^+$, PMS and INT [76,77]. The role of this enzyme, however, can also be extended to the detection of a variety of transaminases in biological systems by coupling the production of glutamate or NH_3 to NAD(P)H formation. For instance, alanine and aspartate transaminase (EC 2.6.1.2 and EC 2.6.1.1, respectively), can both be detected by exposing the gel to the substrates (alanine or aspartate, with KG) and reacting the resultant glutamate with GDH, NAD^+, INT and PMS [78]. Other glutamate and ammonia-generating enzymes, such as ornithine aminotransferase (EC 2.6.1.13), glutaminase (EC 3.5.1.2) and carbamoyl-phosphate synthase (CPS, EC 6.3.4.16) can also be probed as their respective reactions generate a product detected by GDH [78]. The activity of CPS, a crucial component of the urea cycle and pyrimidine synthesis, is visualized via the coupling of NH_3 production to GDH [78]. This reaction is particularly intriguing, as the common protocol for its detection involves the coupling of the enzyme to ornithine transcarbamoylase followed by the colorimetric measurement of citrulline formed [79]. As this method is commonly subject to interference from endogenous compounds in the biological sample, BN-PAGE provides a means to more accurately qualify the activity of this enzyme [80].

5. Multi-protein structures and inter/intra-organellar metabolism

The dynamic nature of biological processes necessitates constant association, dissociation and re-arrangement of the proteins participating in a specific task [6]. These functional effectors may be the residents of the same or disparate organelles. The interactions among partners are ephemeral and are governed by transient forces that are usually amenable to BN-PAGE [13,23]. Indeed, this technique has helped identify the cooperation between proteins and scaffolding networks, metabolons, and trans-organellar communication [31]. For instance, the application of BN-PAGE has revealed 50

hetero-multimeric complexes in *Escherichia coli*, compared to the 18 identified by tandem affinity purification [81]. Some of these superstructures include twin arginine translocase, multidrug efflux transporters, methanogenesis complexes and aromatic degradation complexes [82–86]. In *Pseudomonas fluorescens*, a metabolon generated in response to nitrosative stress and consisting of the metabolic enzymes citrate lyase (CL), PEPC and PPDK has been demonstrated. This complex is promoted by the phosphorylation of PPDK and its dismantling ensues following de-phosphorylation [23]. This enzymatic assembly favours the production of ATP and the channelling of citrate [35]. In yeast, the glycolytic enzyme enolase, which catalyzes the formation of PEP from 2-phosphoglycerate, has been found associated with the mitochondrial membrane [87]. This peripheral localization of a glycolytic enzyme with the mitochondrion was visualized with the aid of BN-PAGE. The unearthing of other components of this multi-enzymatic machine that includes aldolase, GAPDH, mitochondrial porins, ATP synthase subunits, citrate synthase and MDH has been made possible by this electrophoretic technique [87]. Recently, the interaction between LDH and SIRT1 in the nucleus has been demonstrated. In this instance, LDH modulates the concentration of NAD^+, a metabolite intimately associated with the modification of histones and gene regulation [58,88]. Although these MPCs likely contain other candidate proteins that may be sensitive to electrophoresis, it is tempting to speculate that these enzymatic congregations representing disparate metabolic hubs are brought together in order to execute a specific time-sensitive biological function.

6. Conclusion and future perspectives

While genetic microarrays and protein arrays can help discern the overall expression of enzymes involved in various metabolic hubs, these data do not necessarily correlate to the final product or biological outcome. The information acquired by these procedures relates to biomolecules or their products that can be modified or lost depending on cellular conditions [89–91]. Hence, in order to understand the biological action that occurs, the monitoring of enzymatic activities is critical (Suppl. Table 1). The workings of these enzymes need to be captured in a non-invasive manner where the transient assembly and the intimacy of MPCs are maintained [7,23]. BN-PAGE is indeed a powerful tool to image the enzymatic interactions contributing to select biological functions and metabolic networks. The activity bands generated lend themselves to further manipulations such as high performance liquid chromatography (HPLC), two-dimensional SDS-PAGE, and mass spectrometry. These analyses complement the pivotal data on enzymatic activities and their partnerships with other entities in the cell [17,30]. This electrophoretic technique is also conducive to the monitoring of isoenzymes and can be utilized to elucidate the metabolic status of the organism by visualizing key enzymes in select pathways [53]. Hence, it is quite likely that this relatively simple procedure will unearth more tantalizing details on how enzymes conjugate to engineer the workings of metabolic networks [92–94]. These processes that may necessitate cooperation from participants residing in the same or disparate organelles reveal a precise snapshot of cellular operations.

Acknowledgements

This work is funded by Laurentian University and the Northern Ontario Heritage Fund. Azhar Alhasawi is a recipient of funding from the Ministry of Higher Education of Saudi Arabia.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.euprot.2015.05.003.

REFERENCES

[1] Myers MG, Olson DP. Central nervous system control of metabolism. Nature 2012;491:357–63.
[2] Locasale JW. Serine, glycine and one-carbon units: cancer metabolism in full circle. Nat Rev Cancer 2013;13:572–83.
[3] Ghesquiere B, Wong BW, Kuchnio A, Carmeliet P. Metabolism of stromal and immune cells in health and disease. Nature 2014;511:167–76.
[4] Pearce EL, Poffenberger MC, Chang CH, Jones RG. Fueling immunity: insights into metabolism and lymphocyte function. Science 2013;342:1242454.
[5] Friedman JR, Nunnari J. Mitochondrial form and function. Nature 2014;505:335–43.
[6] Winkel BS. Metabolic channeling in plants. Annu Rev Plant Biol 2004;55:85–107.
[7] Williamson MP, Sutcliffe MJ. Protein–protein interactions. Biochem Soc Trans 2010;38:875–8.
[8] Vandooren J, Geurts N, Martens E, Van den Steen PE, Opdenakker G. Zymography methods for visualizing hydrolytic enzymes. Nat Methods 2013;10:211–20.
[9] Seelert H, Krause F. Preparative isolation of protein complexes and other bioparticles by elution from polyacrylamide gels. Electrophoresis 2008;29:2617–36.
[10] Schagger H, von Jagow G. Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal Biochem 1991;199:223–31.
[11] Nijtmans LG, Henderson NS, Holt IJ. Blue native electrophoresis to study mitochondrial and other protein complexes. Methods 2002;26:327–34.
[12] Novakova Z, Man P, Novak P, Hozak P, Hodny Z. Separation of nuclear protein complexes by blue native polyacrylamide gel electrophoresis. Electrophoresis 2006;27:1277–87.
[13] Wittig I, Braun HP, Schagger H. Blue native PAGE. Nat Protoc 2006;1:418–28.
[14] Krause F. Detection and analysis of protein–protein interactions in organellar and prokaryotic proteomes by native gel electrophoresis: (membrane) protein complexes and supercomplexes. Electrophoresis 2006;27:2759–81.
[15] Barglow KT, Cravatt BF. Activity-based protein profiling for the functional annotation of enzymes. Nat Methods 2007;4:822–7.
[16] Dresler J, Klimentova J, Stulik J. Bacterial protein complexes investigation using blue native PAGE. Microbiol Res 2011;166:47–62.
[17] Han S, et al. The unravelling of metabolic dysfunctions linked to metal-associated diseases by blue native polyacrylamide gel electrophoresis. Anal Bioanal Chem 2013;405:1821–31.

[18] Vafai SB, Mootha VK. Mitochondrial disorders as windows into an ancient organelle. Nature 2012;491:374–83.
[19] Saraste M. Oxidative phosphorylation at the fin de siecle. Science 1999;283:1488–93.
[20] Leary SC. Blue native polyacrylamide gel electrophoresis: a powerful diagnostic tool for the detection of assembly defects in the enzyme complexes of oxidative phosphorylation. Methods Mol Biol 2012;837:195–206.
[21] Ohtake A, et al. Diagnosis and molecular basis of mitochondrial respiratory chain disorders: exome sequencing for disease gene identification. Biochim Biophys Acta 2014;1840:1355–9.
[22] Auger C, et al. Metabolic reengineering invoked by microbial systems to decontaminate aluminum: implications for bioremediation technologies. Biotechnol Adv 2013;31: 266–73.
[23] Auger C, Appanna VD. A novel ATP-generating machinery to counter nitrosative stress is mediated by substrate-level phosphorylation. Biochim Biophys Acta 2014;1850:43–50.
[24] Calvaruso MA, Smeitink J, Nijtmans L. Electrophoresis techniques to investigate defects in oxidative phosphorylation. Methods 2008;46:281–7.
[25] Middaugh J, et al. Aluminum triggers decreased aconitase activity via Fe-S cluster disruption and the overexpression of isocitrate dehydrogenase and isocitrate lyase: a metabolic network mediating cellular survival. J Biol Chem 2005;280:3159–65.
[26] Singh R, et al. Blue native polyacrylamide gel electrophoresis and the monitoring of malate- and oxaloacetate-producing enzymes. J Biochem Biophys Methods 2005;64:189–99.
[27] Schreiner ME, Eikmanns BJ. Pyruvate: quinone oxidoreductase from *Corynebacterium glutamicum*: purification and biochemical characterization. J Bacteriol 2005;187:862–71.
[28] Auger C, Appanna V, Castonguay Z, Han S, Appanna VD. A facile electrophoretic technique to monitor phosphoenolpyruvate-dependent kinases. Electrophoresis 2012;33:1095–101.
[29] Han S, Auger C, Thomas SC, Beites CL, Appanna VD. Mitochondrial biogenesis and energy production in differentiating murine stem cells: a functional metabolic study. Cell Reprogram 2014;16:84–90.
[30] Brookes PS, et al. High throughput two-dimensional blue-native electrophoresis: a tool for functional proteomics of mitochondria and signaling complexes. Proteomics 2002;2:969–77.
[31] Shekari F, et al. Identification of cytoplasmic and membrane-associated complexes in human embryonic stem cells using blue native PAGE. Mol Biosyst 2011;7:2688–701.
[32] Balabaskaran Nina P, et al. ATP synthase complex of *Plasmodium falciparum*: dimeric assembly in mitochondrial membranes and resistance to genetic disruption. J Biol Chem 2011;286:41312–22.
[33] Benit P, et al. Unsuspected task for an old team: succinate, fumarate and other Krebs cycle acids in metabolic remodeling. Biochim Biophys Acta 2014;1837:1330–7.
[34] Stark R, Kibbey RG. The mitochondrial isoform of phosphoenolpyruvate carboxykinase (PEPCK-M) and glucose homeostasis: has it been overlooked? Biochim Biophys Acta 2014;840:1313–30.
[35] Auger C, Lemire J, Cecchini D, Bignucolo A, Appanna VD. The metabolic reprogramming evoked by nitrosative stress triggers the anaerobic utilization of citrate in *Pseudomonas fluorescens*. PLoS ONE 2011;6:e28469.
[36] Yan LJ, Sumien N, Thangthaeng N, Forster MJ. Reversible inactivation of dihydrolipoamide dehydrogenase by mitochondrial hydrogen peroxide. Free Radic Res 2013;47:123–33.
[37] Richardson AR, et al. Multiple targets of nitric oxide in the tricarboxylic acid cycle of *Salmonella enterica* serovar typhimurium. Cell Host Microbe 2011;10:33–43.
[38] Yan LJ, Yang SH, Shu H, Prokai L, Forster MJ. Histochemical staining and quantification of dihydrolipoamide dehydrogenase diaphorase activity using blue native PAGE. Electrophoresis 2007;28:1036–45.
[39] Henderson NS, et al. Separation of intact pyruvate dehydrogenase complex using blue native agarose gel electrophoresis. Electrophoresis 2000;21:2925–31.
[40] Lemire J, Auger C, Mailloux R, Appanna VD. Mitochondrial lactate metabolism is involved in antioxidative defense in human astrocytoma cells. J Neurosci Res 2014;92: 464–75.
[41] Boron WF. Evaluating the role of carbonic anhydrases in the transport of HCO_3-related species. Biochim Biophys Acta 2010;1804:410–21.
[42] An S, Kumar R, Sheets ED, Benkovic SJ. Reversible compartmentalization of *de novo* purine biosynthetic complexes in living cells. Science 2008;320:103–6.
[43] Cleaver JE, et al. Mitochondrial reactive oxygen species are scavenged by Cockayne syndrome B protein in human fibroblasts without nuclear DNA damage. Proc Natl Acad Sci 2014;111:13487–92.
[44] Kannan N, et al. Glutathione-dependent and -independent oxidative stress-control mechanisms distinguish normal human mammary epithelial cell subsets. Proc Natl Acad Sci 2014;111:7789–94.
[45] Venditti P, Di Stefano L, Di Meo S. Mitochondrial metabolism of reactive oxygen species. Mitochondrion 2013;13: 71–82.
[46] Reisinger V, Eichacker LA. Organelle proteomics: reduction of sample complexity by enzymatic in-gel selection of native proteins. Methods Mol Biol 2009;564:325–33.
[47] Beriault R, et al. Detection and purification of glucose 6-phosphate dehydrogenase, malic enzyme, and NADP-dependent isocitrate dehydrogenase by blue native polyacrylamide gel electrophoresis. Electrophoresis 2005;26:2892–7.
[48] Fan J, et al. Quantitative flux analysis reveals folate-dependent NADPH production. Nature 2014;510:298–302.
[49] Liang X, Dickman MB, Becker DF. Proline biosynthesis is required for endoplasmic reticulum stress tolerance in *Saccharomyces cerevisiae*. J Biol Chem 2014;289:27794–806.
[50] Sies H. Role of metabolic H_2O_2 generation: redox signaling and oxidative stress. J Biol Chem 2014;289:8735–41.
[51] Bignucolo A, et al. Hydrogen peroxide stress provokes a metabolic reprogramming in *Pseudomonas fluorescens*: enhanced production of pyruvate. J Biotechnol 2013;167:309–15.
[52] Yu Q, et al. Characterization of the NADP-malic enzymes in the woody plant *Populus trichocarpa*. Mol Biol Rep 2013;40:1385–96.
[53] Beriault R, et al. The overexpression of NADPH-producing enzymes counters the oxidative stress evoked by gallium, an iron mimetic. Biometals 2007;20:165–76.
[54] Magni G, Orsomando G, Raffaelli N. Structural and functional properties of NAD kinase, a key enzyme in NADP biosynthesis. Mini Rev Med Chem 2006;6:739–46.
[55] Labesse G, Douguet D, Assairi L, Gilles AM. Diacylglyceride kinases, sphingosine kinases and NAD kinases: distant relatives of 6-phosphofructokinases. Trends Biochem Sci 2002;27:273–5.
[56] Mailloux RJ, Singh R, Appanna VD. In-gel activity staining of oxidized nicotinamide adenine dinucleotide kinase by blue native polyacrylamide gel electrophoresis. Anal Biochem 2006;359:210–5.

[57] Ramzan R, Weber P, Linne U, Vogt S. GAPDH: the missing link between glycolysis and mitochondrial oxidative phosphorylation? Biochem Soc Trans 2013;41:1294–7.
[58] Castonguay Z, Auger C, Thomas SC, Chahma M, Appanna VD. Nuclear lactate dehydrogenase modulates histone modification in human hepatocytes. Biochem Biophys Res Commun 2014;454:172–7.
[59] Lemire J, Mailloux RJ, Appanna VD. Mitochondrial lactate dehydrogenase is involved in oxidative-energy metabolism in human astrocytoma cells (CCF-STTG1). PLoS ONE 2008;3:e1550.
[60] Leyn SA, et al. Control of proteobacterial central carbon metabolism by the HexR transcriptional regulator: a case study in *Shewanella oneidensis*. J Biol Chem 2011;286:35782–94.
[61] Deramchia K, et al. Contribution of pyruvate phosphate dikinase in the maintenance of the glycosomal ATP/ADP balance in the *Trypanosoma brucei* procyclic form. J Biol Chem 2014;289:17365–78.
[62] Saidin S, et al. Production of recombinant *Entamoeba histolytica* pyruvate phosphate dikinase and its application in a lateral flow dipstick test for amoebic liver abscess. BMC Infect Dis 2014;14:182.
[63] Wang Q, Ingram LO, Shanmugam KT. Evolution of D-lactate dehydrogenase activity from glycerol dehydrogenase and its utility for D-lactate production from lignocellulose. Proc Natl Acad Sci 2011;108:18920–5.
[64] Swamy M, et al. Detection of protein complex interactions via a Blue Native-PAGE retardation assay. Anal Biochem 2009;392:177–9.
[65] Menefee AL, Zeczycki TN. Nearly 50 years in the making: defining the catalytic mechanism of the multifunctional enzyme, pyruvate carboxylase. FEBS J 2014;281:1333–54.
[66] Carlson GM, Holyoak T. Structural insights into the mechanism of phosphoenolpyruvate carboxykinase catalysis. J Biol Chem 2009;284:27037–41.
[67] Izui K, Matsumura H, Furumoto T, Kai Y. Phosphoenolpyruvate carboxylase: a new era of structural biology. Annu Rev Plant Biol 2004;55:69–84.
[68] Russell DG, Cardona PJ, Kim MJ, Allain S, Altare F. Foamy macrophages and the progression of the human tuberculosis granuloma. Nat Immunol 2009;10:943–8.
[69] Hamel R, Appanna VD, Viswanatha T, Puiseux-Dao S. Overexpression of isocitrate lyase is an important strategy in the survival of *Pseudomonas fluorescens* exposed to aluminum. Biochem Biophys Res Commun 2004;317:1189–94.
[70] Strable MS, Ntambi JM. Genetic control of *de novo* lipogenesis: role in diet-induced obesity. Crit Rev Biochem Mol Biol 2010;45:199–214.
[71] Mailloux R, Lemire J, Appanna V. Aluminum-induced mitochondrial dysfunction leads to lipid accumulation in human hepatocytes: a link to obesity. Cell Physiol Biochem 2007;20:627–38.
[72] Nissim I, et al. The molecular and metabolic influence of long term agmatine consumption. J Biol Chem 2014;289:9710–29.
[73] Lemire J, Mailloux R, Darwich R, Auger C, Appanna VD. The disruption of L-carnitine metabolism by aluminum toxicity and oxidative stress promotes dyslipidemia in human astrocytic and hepatic cells. Toxicol Lett 2011;203:219–26.
[74] Eubel H, Braun HP, Millar AH. Blue-native PAGE in plants: a tool in analysis of protein–protein interactions. Plant Methods 2005;1:11.
[75] Gunka K, Commichau FM. Control of glutamate homeostasis in *Bacillus subtilis*: a complex interplay between ammonium assimilation, glutamate biosynthesis and degradation. Mol Microbiol 2012;85:213–24.
[76] Lemire J, et al. Histidine is a source of the antioxidant, alpha-ketoglutarate, in *Pseudomonas fluorescens* challenged by oxidative stress. FEMS Microbiol Lett 2010;309:170–7.
[77] Mailloux RJ, et al. Alpha-ketoglutarate dehydrogenase and glutamate dehydrogenase work in tandem to modulate the antioxidant alpha-ketoglutarate during oxidative stress in *Pseudomonas fluorescens*. J Bacteriol 2009;191:3804–10.
[78] Han S, et al. A blue native polyacrylamide gel electrophoretic technology to probe the functional proteomics mediating nitrogen homeostasis in *Pseudomonas fluorescens*. J Microbiol Methods 2012;90:206–10.
[79] Nakagawa T, Lomb DJ, Haigis MC, Guarente L. SIRT5 Deacetylates carbamoyl phosphate synthetase 1 and regulates the urea cycle. Cell 2009;137:560–70.
[80] Ahuja A, Purcarea C, Guy HI, Evans DR. A novel carbamoyl-phosphate synthetase from *Aquifex aeolicus*. J Biol Chem 2001;276:45694–703.
[81] Lasserre JP, et al. A complexomic study of *Escherichia coli* using two-dimensional blue native/SDS polyacrylamide gel electrophoresis. Electrophoresis 2006;27:3306–21.
[82] Baglieri J, Beck D, Vasisht N, Smith CJ, Robinson C. Structure of TatA paralog, TatE, suggests a structurally homogeneous form of Tat protein translocase that transports folded proteins of differing diameter. J Biol Chem 2012;287:7335–44.
[83] Ferrandez Y, et al. Stoichiometry of the MexA-OprM binding, as investigated by blue native gel electrophoresis. Electrophoresis 2012;33:1282–7.
[84] Farhoud MH, et al. Protein complexes in the archaeon *Methanothermobacter thermautotrophicus* analyzed by blue native/SDS-PAGE and mass spectrometry. Mol Cell Proteomics 2005;4:1653–63.
[85] Reig N, et al. Functional and structural characterization of the first prokaryotic member of the L-amino acid transporter (LAT) family: a model for APC transporters. J Biol Chem 2007;282:13270–81.
[86] Tsirogianni E, Aivaliotis M, Papasotiriou DG, Karas M, Tsiotis G. Identification of inducible protein complexes in the phenol degrader Pseudomonas sp. strain phDV1 by blue native gel electrophoresis and mass spectrometry. Amino Acids 2006;30:63–72.
[87] Brandina I, et al. Enolase takes part in a macromolecular complex associated to mitochondria in yeast. Biochim Biophys Acta 2006;1757:1217–28.
[88] Choudhary C, Weinert BT, Nishida Y, Verdin E, Mann M. The growing landscape of lysine acetylation links metabolism and cell signalling. Nat Rev Mol Cell Biol 2014;15:536–50.
[89] Wang Q, et al. Acetylation of metabolic enzymes coordinates carbon source utilization and metabolic flux. Science 2010;327:1004–7.
[90] Torres AG, Batlle E, Ribas de Pouplana L. Role of tRNA modifications in human diseases. Trends Mol Med 2014;20:306–14.
[91] Helm M, Alfonzo JD. Posttranscriptional RNA modifications: playing metabolic games in a cell's chemical Legoland. Chem Biol 2014;21:174–85.
[92] Mattila S, Oksanen HM, Bamford JK. Probing of protein interactions in the membrane-containing virus PRD1. J Gen Virol 2014;14:187–90.
[93] Nakamura M, et al. Proteomic analysis of protein complexes in human SH-SY5Y neuroblastoma cells by using blue-native gel electrophoresis: an increase in lamin A/C associated with heat shock protein 90 in response to 6-hydroxydopamine-induced oxidative stress. Exp Gerontol 2009;44:375–82.
[94] Phillips D, Aponte AM, Covian R, Balaban RS. Intrinsic protein kinase activity in mitochondrial oxidative phosphorylation complexes. Biochemistry 2011;50:2515–29.