Summary of the Esselen Award Address

The New Generation Chemistry for Newborn Screening

By Michael H. Gelb and František Tureček, Department of Chemistry, University of Washington, Seattle

Diseases caused by inborn errors of metabolism represent a diverse group of several thousand known syndromes (*1*). Some affect specific organs, some are systemic, and their effects range from mild that appear in adolescence or adulthood, to severe that, if untreated, result in an early death of affected children. Inborn errors of metabolism are rare on an individual basis, but their combined prevalence can be as high as one in a few thousand newborns. In the past decade, breakthrough progress has been made by U.S. pharmaceutical companies in developing sophisticated therapies, such as those based on enzyme replacement or erythropoietic stem cell transplantation, to treat metabolic diseases and save lives of affected children. The new therapies are expensive and carry inherent risks, which places extremely stringent criteria on diagnostic accuracy. Detection of inborn errors poses specific problems, as the disorder phenotype may not be obvious in the early stages of the disease to apply the suitable therapy. Therefore, most disorders are detected by chemical or bioanalytical methods using blood samples from newborns. Even the use of bioanalytical methods is not without problems. For example, detection of specific low-level proteins in blood is made difficult by interferences from high-abundance blood proteins such as albumin. DNA analysis is hampered by the genetic variability of mutations leading to the disorder. Detection of disease-specific metabolites can be problematic because of their low levels in blood and interferences from other, chemically very similar, blood components. This leaves enzyme activity analysis as a powerful and very promising chemical approach in which specific enzymes are targeted, and the lack of their activity signals a metabolic error.

The principle of enzyme activity measurements is quite simple. A biological sample from the patient is incubated in vitro with a synthetic substrate which is recognized and chemically modified by the target enzyme. The product of the enzyme-catalyzed reaction is then quantitatively analyzed and used to calculate the enzyme activity, which is compared to the mean activity measured for samples from many others, typically hundreds of individuals. If the activity is below a statistically determined cutoff value, the sample is flagged for follow up genotype analysis to determine the DNA mutation. The analytical procedure must have figures of merit showing good reproducibility, high specificity, and a very low rate of false positives. In addition, in order to be applied in a large scale format of newborn screening, the enzyme activity measurements must be fast and inexpensive. In our initial approach, we used a combination of mass spectrometry and affinity purification to develop chemical methods of enzyme activity measurements that satisfied the first three figures of merit. To improve speed and throughput, we turned to tandem mass spectrometry to develop new generation methods for newborn screening of inborn errors of metabolism.

The diseases we have focused on comprised lysosomal storage disorders (LSD), congenital disorders of glycosylation (CDG), and porphyrias. The first and most important group includes disorders affecting activities of ca. 50 hydrolytic enzymes that catalyze degradation of biopolymers in the lysosome. CDG types I-VII are a group of disorders affecting activities of enzymes catalyzing carbohydrate synthesis and protein N-glycosylation in the endoplasmic

reticulum. Porphyrias are rare diseases caused by deficient enzymes in the eight-step long biosynthesis of the heme that proceeds in part in the cytosol and in part in mitochondria of liver and bone marrow cells.

The synthetic enzyme substrates were designed and made to include a structure moiety recognized by the enzyme. Most of the LSD substrates include carbohydrate moieties (glucose, galactose, N-acetyl galactosamine, a- L-iduronic acid, etc.) with a or b glycosidic linkages. Several LSD enzymes are sulfatases that recognize O- or N-sulfate groups in particular positions of the sugar molecules. For example, mucopolysaccharidosis III types A and D (also known as Sanfilippo syndromes) are caused by deficiency of enzymes degrading the glycosaminoglycan heparan sulfate by selectively stripping sulfate groups from glucosamine-N-sulfate and Nacetylglucosamine-6-O-sulfate units that are linked by a-glycosidic bonds to glucuronic acid. The synthetic substrates must encompass these structure features to be recognized by the enzymes. Another example concerns the two most common forms of CDG caused by deficient enzymes phosphomannoisomerase (PMI) and phosphomannomutase isozyme 2(PMM2). PMI works on converting fructose-6-phosphate to its isomer mannose-6-phosphate to be converted by PMM2 to another isomer, mannose-1-phosphate, which is then used to build glycan antennas in glycoproteins. Distinguishing these isomeric molecules by mass spectrometry was quite challenging and required a special strategy of coupled enzyme assays to succeed.

Quantitative analysis of enzyme activities by mass spectrometry relies on the simultaneous determination of enzyme products and internal standards. The latter are synthetic compounds that are chemically very similar to or identical with the enzyme products but are distinguished by molecular mass owing to the presence of stable heavy isotopes or homologous groups. The chemical similarity of products and internal standards ensures that the response of the mass spectrometer to these compounds is also very similar which simplifies analysis.

Enzyme assays are carried out in biological samples that are very complex mixtures of many compound types (DNA, proteins, lipids, saccharides) and include exogenous components such as buffer salts, detergents, inhibitors, and other additives. This mandates that after incubation the assay sample be purified to exclude components that would interfere with ionization and mass spectrometric analysis. In our initial approach, we used affinity purification based on the highly specific and reversible non-covalent biotin-streptavidin interaction (*2*). Substrates and internal standards were synthesized that contained a covalently linked biotin moiety that served as an affinity handle for affinity purification. This approach, in combination with stable isotope labeling, was extended by collaborators to protein analysis using in vitro derivatization, proteolysis, and bottom-up peptide quantification. This method, called Isotope-Coded Affinity Tags (ICAT for short), was introduced in 1999 and pioneered quantitative protein analysis in the then burgeoning field of proteomics (*3*).

Affinity purification with streptavidin immobilized on polymer beads, followed by mass spectrometry analysis, was suitable for applications in clinical diagnostics and was semi-automated using a lab-on-valve apparatus for bead injection that was coupled to the mass spectrometer.

Enzyme assay development involved a thorough analysis of enzyme kinetics and optimization of

experimental conditions. Lysosomal enzymes work in an acidic environment of pH 4.0-4.5 and so the pH dependence of their in vitro activity for the synthetic substrates had to be established. The substrate conversion was purposefully limited to a few percent to maintain pseudo first order kinetics and linear time dependence of product formation. Linear dependence on the substrate and enzyme concentration was also tested. Interfering enzymes present in the biological sample were suppressed by specific inhibitors.

In 2001, the late Nestor Chamoles reported that lysosomal enzymes retain latent activity in dried blood spots and can be assayed after rehydration in a suitable buffer (*4*). This discovery caused a major change in the direction of our research of LSD that ever since has focused on dried blood spots (DBS)(*5*). Since DBS are used as a common biological sample in newborn screening, our efforts were focused on retooling our chemistry of substrates and internal standards, bioanalytical work up procedures, and mass spectrometric analysis. Particular emphasis has been placed on multiplexing all these steps so that multiple enzyme activities could be determined simultaneously in one or two parallel DBS incubations. Our new generation substrates were designed to comprise three major parts: (i) a structure moiety that is recognized by the enzyme, (ii) a group allowing facile introduction of stable isotope label, and (iii) a functional group directing the ion fragmentation in the mass spectrometer into one dominant channel producing the reporter ion. At the same time, the compounds were designed such that the enzyme reaction products were readily separated from the incubation medium by a single step procedure, e.g., extraction or ultrafast chromatography. As of now, we have developed substrates and procedures for 15 lysosomal enzymes, galactocerebroside bgalactosidase (GALC), acid sphingomyelinase (ASM), acid b-glucocerebrosidase (ABG), acid a-galactosidase (GLA), acid a-glucosidase (GAA), a-L-iduronidase (IDUA), iduronate-2-sulfatase (IdS), N-acetylgalactosamine- 4-sulfatase (aryl sulfatase B, ASB), N-acetylgalactosamine- 6-sulfate sulfatase (GALNS), heparan N-sulfatase (sulfamidase), α-N-acetylglucosaminidase, acetyl-CoA:α-glucosaminide acetyltransferase, Nacetylglucosamine-6-sulfatase, palmitoyl protein thioesterase (PPT), and tripeptidyl peptidase 1 (TPP1). The first six of these enzymes have been included in a six-plex procedure that is being tested in a pilot program of newborn screening in Illinois. A pilot study of a triplex assay (GLA, GAA and IDUA) has been successfully carried out with >100,000 samples in Washington state (*6*). A diagnostic newborn screening of GALC deficiency (Krabbe disease) has been running in New York state that has so far included over 1,000,000 samples. Further technological advances pursued in our laboratory include ultra-fast high performance liquid chromatography for sample purification after incubation (*7*) and design of new substrates.

References

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