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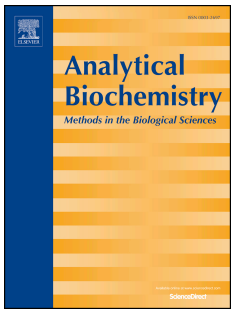

Native denaturation differential scanning fluorimetry: Determining the effect of urea using a quantitative real-time thermocycler

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Native denaturation differential scanning fluorimetry: determining the effect of urea using a quantitative real-time thermocycler.

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Abstract: The effect of protein stability on kinetic function is monitored with many techniques that often require large amounts of expensive substrates and specialized equipment not universally available. We present differential scanning fluorometry (DSF), a simple high-throughput assay performed in real-time thermocyclers, as a technique to analyze protein unfolding. Furthermore, we demonstrate a correlation between the half maximal rate of protein unfolding (K_{nd}), and protein unfolding by urea (I_{50}). This demonstrates that DSF methods can determine the structural stability of an enzyme's active site and can compare the relative structural stability of homologous enzymes with a high degree of sequence similarity.

Homologous enzymes that catalyze identical reactions in the same organism, known as isozymes, provide the unique opportunity to study natural functional changes in enzymatic activity due to very little difference in primary structure. The lactate dehydrogenase (LDH) family of enzymes is one of the earliest, and well characterized, examples of the use of isozymes to provide insight into the relationship between structure and function (Read et al., 2001). LDH is a tetramer with each subunit belonging to one of two major forms: the M (muscle) form which is found primarily in skeletal muscle and liver, and the H (heart) form which is found primarily in cardiac muscle. LDH provides an excellent example of the biological utility of multimeric proteins, insofar that a relatively small number of subunit genes can lead to a diverse repertoire of isozymes, each uniquely fitted to the specific needs of multiple tissues. The isozymes examined in this study were derived from heart tissue (4H isozyme) and skeletal muscle (4M isozyme) and therefore represent homotetramers. Importantly, Read et al. (2010) noted that there is a preservation of the active-site features between these isozymes with the altered kinetic properties due to a single amino acid, His 195.

Bioinformatic analysis of subtle changes in the amino acid sequence of a protein can predict optimal protein stability, however determination of the biophysical effects on the enzymes is less clear using these predictive methods. Traditionally the stability of proteins in solution was estimated from temperature-induced unfolding by using 2 different methods: differential scanning calorimetry and monitoring the changes in ellipticity at 222 nm by using circular dichroism spectroscopy. While both of these techniques have some value in determining protein stability, they suffer from being lower throughput than DSF and involve instruments that might not be ubiquitously found in molecular biology laboratories (Vollrath et al., 2014). The differential scanning fluorimetry technique has recently provided a novel mechanism for determining temperature stability using a quantified real-time thermocycler (Niesen et al., 2007). Furthermore, a modification to this technique, whereby fluorescence is monitored during protein unfolding in the presence of increasing amounts of urea or other denaturants, allows for a more precise indication of changes to the proteins surface structure (Biggar et al, 2012). Using both DSF techniques in combination with kinetic analysis we aim to demonstrate the effectiveness of this technique in resolving the core and surface stability differences between two proteins with high sequence similarity: the 4H tetramer from