Activity Measurements of Pentose Phosphate Pathway Enzymes Using the Thermo Scientific NanoDrop 2000c Kinetics Application

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Key Words

E. coli, NanoDrop 2000c, Nuclear Magnetic Resonance (NMR), Pentose Phosphate Enzyme, Ribonucleic Acid (RNA), Ribokinase, UV-Visible Spectrophotometry

Introduction

In the last decade, it has become clear that Ribonucleic Acid (RNA) is a major player in the central dogma of biology. Therefore, the biochemistry and structural biology of RNA are of great interest to the scientific community, especially as potential drug targets.[1-5] Currently, there are few effective methods for solving the three-dimensional structures of RNAs, with Nuclear Magnetic Resonance (NMR) spectroscopy being one of the most robust.[6] However, NMR structure elucidation of RNAs ≥30 nucleotides faces two formidable challenges: extensive spectral overcrowding and linewidth broadening.[7] One of the approaches to solving these problems is the use of site-specifically ^{13}C and ^{15}N-labeled nucleotides incorporated into the RNA of interest.

A method developed by Arthur et al. describes the use of several enzymes from the *E. coli* pentose phosphate pathway to synthesize such nucleotides from both ribose and the corresponding nucleobase.[8] In this work, six enzymes were expressed, purified and characterized through a straightforward protocol, yielding specific activities comparable to or greater than those previously described. The biochemical characterization of these consisted of a series of direct or indirect continuous spectrophotometric assays according to previously established protocols.[9-15]

Traditionally, these assays are monitored by large UV-Vis spectrophotometers requiring substantial bench space. However, the Thermo Scientific™ NanoDrop™ 2000c spectrophotometer, at a fraction of the size, allows for accurate measurements of changes in absorbance at various wavelengths in real time by means of its novel Kinetics Module. This module allows the experimenter to monitor absorbance over a period of time as needed by the experimenter (i.e. experiment length, reading frequency and interval). Additionally, this instrument allows for measurements to be carried out in 1 mL cuvettes with the options to incubate the sample at 37 °C and perform stirring throughout the assay. This technical note briefly summarizes one example of the experimental protocol used and the results obtained by Arthur et al., who use continuous spectrophotometric assays to measure the activity of several enzymes.[8] Here, the *E. coli* enzyme ribokinase (E.C. 2.7.1.15) is used as an example.

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Experimental Procedures

Briefly, the gene coding for ribokinase (RK) was subcloned into a pET15b plasmid (Novagen) and transformed into overexpressing *E. coli* strain BL21(AI) (Invitrogen). *E. coli* was grown in Luria-Bertani broth supplemented with 100 µg/mL ampicillin and RK overexpression was induced by 0.05% L-(+)-arabinose followed by 1 mM isopropyl β-D-1-thiogalactopyranoside. The harvested cells were frozen overnight and then lysed to recover RK. The cell pellet was resuspended, treated with lysozyme, sonicated and then clarified by ultracentrifugation. Then, the whole cell lysate was subjected to Nickel-Nitrilotriacetic acid affinity chromatography, from which >90% pure RK was recovered. RK was then concentrated and buffer exchanged (50 mM NaH_2PO_4 (pH 7.5), 150 mM NaCl). RK was assayed by an indirect continuous spectrophotometric assay, previously described.[9] The assay schematic is shown in Figure 1. For the detailed procedure, refer to Arthur et al.[8]

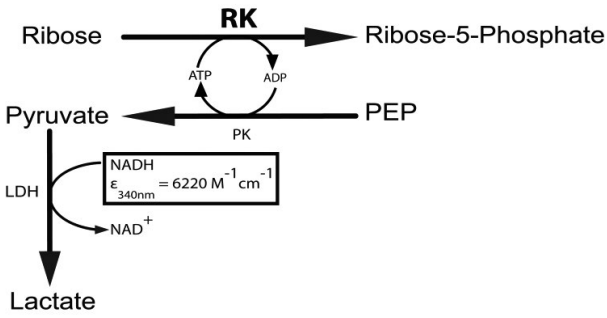

Figure 1: Indirect continuous assay used for ribokinase characterization. The oxidation of NADH is monitored throughout the assay via its absorption decrease at 340 nm. PK: pyruvate kinase, LDH: lactate dehydrogrenase, PEP: phosphoenol pyruvate. Modified from Reference 6.

The assay mixture (1 mL) contained 50 mM Tris-HCl buffer (pH 7.8), 5 mM Ribose, 3 mM ATP, 1 mM PEP, 100 mM KCl, 10 mM $MgCl_2$, 0.2 mM NADH, 2 U of lactate dehydrogenase, and 2 U of pyruvate kinase. The mixture was incubated for 5 minutes at room temperature and then a 2 µL aliquot of ribokinase was added to initiate the reaction. The absorbance change at 340 nm (ΔA) was monitored as a function of time. Units of activity (U) were calculated as follows:

$$U = \frac{V}{\varepsilon * l} * \left(\Delta A / t\right)$$

Where, V is the total reaction volume in L, ε is the extinction coefficient of 6220 M^{-1} cm^{-1} at 340 nm for oxidation of NADH to NAD^+, l is the pathlength in cm, ΔA is the change in absorbance at 340 nm, and t is the assay time in minutes. Using the rate vector function in the NanoDrop 2000c interface, ΔA/t is obtained. The activity is finally expressed in U (µmol/min).

Results

The enzymatic activity of RK was determined by an indirect continuous spectrophotometric assay. The equation above was used to calculate the activity in three independent assays. Figure 2 shows a screenshot of the Kinetics Module in the NanoDrop 2000c user interface. In Figure 2A, the rate vector function (blue) was used to determine ΔA/t over the range shown.

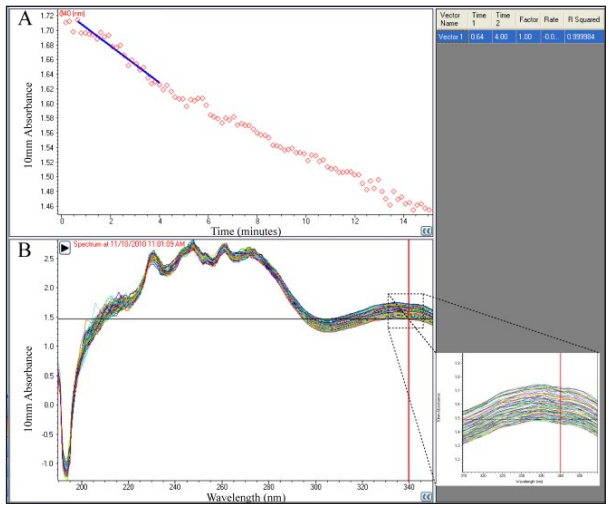

Figure 2: NanoDrop 2000c Kinetics Module interface showing a representative ribokinase assay. (A) Top window: absorbance value at a given time point, with the rate vector in blue. Right Panel: results from rate vector calculations, providing the rate and R squared values. (B) Bottom window: absorbance pattern (200–350 nm) at every time point in the reaction. Inset: Blowout of the region of interest centered at 340 nm.

The results are given on the right side of the window expressed as "Rate" and "R Squared." Figure 2B shows a superimposition of the spectra for each measurement, giving a clear visual indication of the decrease in absorbance at 340 nm over time, (magnified in Figure 2B inset). Once the RK activity was calculated, a progress curve was built according to the stoichiometry of the overall reaction, i.e. one ribose-5-phosphate molecule produced per NADH oxidized. A representative progress curve, from Arthur et al., is shown in Figure 3. The calculated activity of RK was 1530 ± 62 U and its specific activity was 70.2 ± 5.3 U/mg of RK.[8]

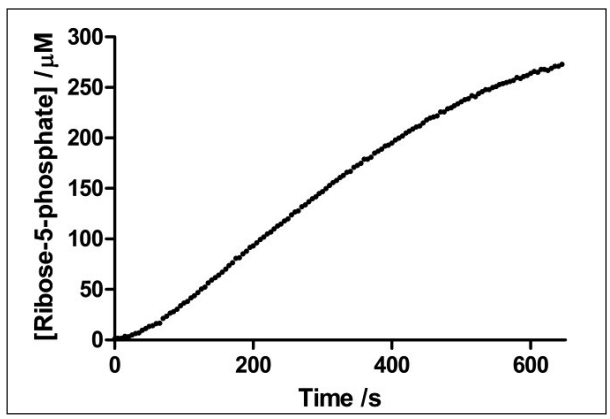

Figure 3: Ribokinase enzymatic assay progress curve. Amount of ribose-5-phosphate made as a function of the reaction time, as calculated from the reaction's stoichiometry. Data are from Reference 6.

Conclusion

The method described in this application note demonstrates the ease of performing enzymatic activity measurements by continuous spectrophotometric assays. Here, an enzyme from the pentose phosphate pathway, ribokinase, was characterized as was previously shown.[8, 9] The activity results were in perfect agreement with previous literature and thus attest to the reliability of the NanoDrop 2000c as a kinetics-measuring instrument.[9]

Additionally, the instrument's Kinetics Module allows for automatic data acquisition to meet the experimenter's needs. Furthermore, the rate vector function calculated the ΔA/t for the specified time interval by using a linear regression analysis, providing a quality R squared value to assess the reliability of the resulting rate. Consequently, both the instrument and module can be used in a flexible manner to meet the needs for a particular enzymatic activity assay.

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