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NMR for direct determination of K_m and V_{max} of enzyme reactions based on the Lambert W function-analysis of progress curves

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ABSTRACT

^{1}H NMR spectroscopy was used to follow the cleavage of sucrose by invertase. The parameters of the enzyme's kinetics, K_m and V_{max}, were directly determined from progress curves at only one concentration of the substrate. For comparison with the classical Michaelis–Menten analysis, the reaction progress was also monitored at various initial concentrations of 3.5 to 41.8 mM. Using the Lambert W function the parameters K_m and V_{max} were fitted to obtain the experimental progress curve and resulted in $K_m = 28$ mM and $V_{max} = 13$ μM/s. The result is almost identical to an initial rate analysis that, however, costs much more time and experimental effort. The effect of product inhibition was also investigated. Furthermore, we analyzed a much more complex reaction, the conversion of farnesyl diphosphate into (+)-germacrene D by the enzyme germacrene D synthase, yielding $K_m = 379$ μM and $k_{cat} = 0.04$ s^{-1}. The reaction involves an amphiphilic substrate forming micelles and a water insoluble product; using proper controls, the conversion can well be analyzed by the progress curve approach using the Lambert W function.

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1. Introduction

Enzyme kinetics is important to understand the mechanisms of enzyme reactions. Many spectroscopic methods are applied frequently in enzyme kinetics. The powerful technique, NMR spectroscopy, by which substrates and products can directly be quantified, is, however, rarely used. Textbooks dealing with enzyme kinetics usually mention NMR spectroscopy only incidentally [1,2]. On the other hand NMR spectroscopic investigations of binding, dynamical and structural properties of enzymes have become quite popular in recent years [3].

Kinetic analysis of enzyme reactions by NMR has some advantages. Measurements are performed in homogenous solutions and the reaction progress can be monitored directly without labeling of the substrate(s). In contrast, relative large sample amounts are necessary for NMR spectroscopy compared to other sensitive standard assays e.g. using fluorescence or radioactive labeling. The requirements on sample quantity were, however, significantly reduced by technical developments in high resolution NMR in the last decade [4,5].

Michaelis–Menten kinetics correlates the initial velocity with the initial substrate concentration of an enzyme reaction. This approach assumes a quasi-steady state approximation where the concentration of the enzyme substrate complex is constant over time and produces a time-independent hyperbolic relation of the two. Since its introduction in 1913, including the Briggs–Haldane modification in 1925, the Michaelis–Menten model has been widely used to describe enzyme processes [6,7]. It has been proven to be a simple yet powerful approach in the determination of enzyme parameters K_m and V_{max}. However, measuring initial velocities at different concentrations by NMR spectroscopy or other analytical techniques is a time and material intensive procedure.

An alternative approach is based on progress curve analysis, where the concentration of the products and/or substrates is followed over time at only one concentration in a single automated experiment [8–10]. This method requires the integrated form of the Michaelis–Menten equation that is implicit in the substrate concentration and the calculation requires numerical integration. This is followed by an appropriate nonlinear optimization routine for an iterative estimation of the kinetic parameters. Later, in 1997 Schnell and Mendoza derived a closed form solution of the integrated Michaelis-Menten equation [11]. Starting from the integrated form they obtained an expression for the substrate concentration as a function of time (Eq. (1)). The solution is based on the Lambert W Function (also called Omega Function) which is defined as the inverse of $x\,e^x$, that is, $W(x) + \ln\{W(x)\} = \ln(x)$ [12]. It was shown by Goudar et al. and others that the Lambert W Function allows an analytical solution of enzyme kinetics from single progress curves [13–15]. The function is implemented in mathematical computing

Abbreviations: FDP, farnesyl diphosphate; (+)-GDS, (+)-germacrene D synthase

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software and can be solved by nonlinear optimization routines (e.g. Maple®, MATLAB®, R).

$$[S] = K'_m W \left\{ \frac{[S]_0}{K'_m} \exp\left(\frac{[S]_0 - V'_{max} t}{K'_m} \right) \right\} \quad (1)$$

Progress curve analysis of enzyme reactions has the advantage that the reaction progress is monitored over the full reaction time and that not only initial reaction rates are monitored. Under these conditions product inhibition can't be neglected and if present influences the values of K_m and V_{max}. For that reason the apparent values K'_m and V'_{max} are obtained in Eq. (1). Another advantage of progress curve analysis originates from the fact that product inhibition can be easily detected by recording progress curves at two initial concentrations. Many aspects of the analysis of enzyme progress curves using non-NMR techniques were summarized by Duggleby [10]. NMR spectroscopy offers the easiest sample handling as well as a high sensitivity. Also, often the direct analysis of the stereochemistry of the initial reactions products can be determined (cf. below). We have chosen the enzyme invertase (*Saccheromyces cerevisea*) used by Michaelis and Menten in order to derive their kinetic model. Invertase hydrolyzes sucrose into glucose and fructose (invert sugar) [6].

2. Materials and methods

2.1. Chemicals

Invertase (EC 3.2.1.26, ß-fructofuranosidase, *S. cerevisea*) was obtained from Sigma-Aldrich (Steinheim, Germany) with a specific activity of 200–300 u/mg enzyme (pH 4.6, 298 K). Sucrose and farnesyl diphosphate were purchased from Sigma-Aldrich (Steinheim, Germany). Glucose and fructose were purchased from Merck (Darmstadt, Germany). D_2O was obtained from Deutero (Kastellaun, Germany), DMSO-d_6 and Tris–HCl-d_{11} from Eurisotop (Saarbrücken, Germany).

2.2. Purification of (+)-germacrene D synthase

(+)-Germacrene D synthase (EC4.2.3.22, sesquiterpene synthase, *Solidago canadensis*) was obtained by heterologous expression in *E. coli* host strain BL21(DE3)pLysS (containing a N-terminal 6-times histidine-tag) as previously described except that after adding IPTG incubation was performed over night at 16 °C [16]. The enzyme was purified from the culture medium by affinity chromatography Ni-NTA-agarose (Qiagen, Hilden, Germany) according to the manufacturers' recommendations. After purification the elution buffer was exchanged immediately with a deuterated tris buffer (50 mM Tris–HCl-d_{11}, 300 mM NaCl, 20 mM NaN_3, D_2O, pH 7.8) using Vivaspin centrifugal concentrators (MWCO 10 kDa, GE Health Care, Freiburg, Germany).

2.3. NMR spectroscopy

All NMR experiments were performed at 298 K (invertase) or 285 K (germacrene D synthase) using a Bruker Avance 700 MHz NMR spectrometer with a 5 mm inverse triple resonance probe head. Spectra were recorded with a spectral width of 9763 Hz (invertase) and 64k data points or 7000 Hz (germacrene D synthase) and 28 k data points. Before the performance of kinetic measurements the NMR magnet was shimmed using a protein sample in the same buffer that was used later (1.5 µg/mL invertase in 25 mM acetate buffer, 50 mM NaCl, 2 mM NaN_3, D_2O, pH 5.0; 2.5 µM germacrene D synthase in 50 mM tris-d_{11}, 300 mM NaCl, 20 mM NaN_3, D_2O, pH 7.8). The spectrometer was matched and tuned and the sample removed. The procedures for sample preparation and data acquisition are described in the following section. The spectra were analyzed with TOPSPIN 2.1 (Bruker). FIDs were treated with 0.5 Hz exponential line-broadening function and were zero-filled once. Integration of signals was performed using the *intser* function of TOPSPIN.

2.4. Preparation of invertase solutions and data acquisition

A sucrose stock solution (3.6 M in 25 mM acetate buffer, 50 mM NaCl, 2 mM NaN_3, D_2O, pH 5.0) was added to a solution of invertase provided in an 2 mL Eppendorf tube (to yield 600 µL with 2.5 µg invertase in 25 mM acetate buffer, 50 mM NaCl, 2 mM NaN_3, D_2O, pH 5.0) and thoroughly mixed using an Eppendorf pipette. Then the reaction mixture was transferred to a 5 mm NMR tube. After the insertion of the NMR tube to the magnet the sample was locked and the experiment started. Eventually the shim was corrected. Spectra were recorded every 2 min applying 8 scans (34.4 s) and using 64, 72 or 180 transients in a pseudo 2D pulse sequence. The acquisition time (AQ) was 3.35 s and a relaxation delay (D1) of 1 s was applied. The initial concentrations of sucrose were 3.5, 8.6, 12.4, 14.8, 15.1 17.8, 22.2, 27.4 and 41.8 mM. For the investigation of product inhibition two samples were equally prepared as described above, except that one sample additionally contained 9 µL of a 1:1 mixture of glucose/fructose (1.8 M in 25 mM acetate buffer, 50 mM NaCl, 2 mM NaN_3, D_2O, pH 5.0).

2.5. Preparation of germacrene D synthase solutions and data acquisition

10 µL of a farnesyl diphosphate stock solution (10 mM FDP in 50 mM tris-d_{11}, 300 mM NaCl, 20 mM NaN_3, D_2O, pH 7.8) was directly added to 190 µL of a solution of (+)-germacrene D synthase provided in a 3 mm NMR tube to give a final volume of 200 µL containing 2.5 µM germacrene D synthase (corresponding to 0.16 mg/mL) in deuterated TBS (50 mM tris-d_{11}, 300 mM NaCl, 20 mM NaN_3, D_2O, pH 7.8, 5% DMSO-d_6 (*v*/*v*), 1 mM $MgCl_2$). The reaction solution was mixed by shaking the NMR tube thoroughly. After insertion of the NMR tube to the magnet the sample was locked, eventually the shim corrected and the experiment started. Spectra were recorded using a pseudo 2D pulse sequence and applying 128 scans on 32 sequential experiments. This pulse sequence contained the excitation sculpting sequence for water suppression. Each single experiment had a total acquisition time of 21 min 55 s.

2.6. Micelle formation of farnesyl diphosphate

A sample was prepared containing 500 µM FDP in deuterated tris buffer (50 mM tris-d_{11}, 300 mM NaCl, 20 mM NaN_3, D_2O, pH 7.8, 5% DMSO-d_6) and a ^1H NMR spectrum was measured (Fig. S6a, Supplementary data). 1 mM $MgCl_2$ was added to the same sample and the experiment was repeated (Fig. S6b, Supplementary data). STD NMR spectra were recorded using a spectral width of 7000 Hz and 32 k time domain data points. The on resonance pulse was set to 5250 Hz, the off resonance pulse to 40 kHz. Saturation was achieved by a train of 90° Gaussian-shaped pulses of 50 ms yielding a total saturation time of 3 s with an attenuation of 45 dB. Water suppression was achieved using the excitation sculpting sequence. O1 was set on resonant to the water signal at 3285 Hz. The temperature during acquisition was 300 K.

2.7. Analysis of invertase reaction

For linear regressions and calculation of Michaelis–Menten kinetics the software OriginPro 8.5.0G SR1 (OriginLab Corporation, Northampton, MA, USA) was used. Progress curves were fitted in MATLAB 7.10.0.499 (R2010a) (MathWorks, Inc., Germany). Initial velocities were plotted against initial substrate concentrations and the hyperbolic curve was fitted according to the one site binding model (pharmacokinetics,

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