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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.

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,

OriginPro) that is equivalent to the Michaelis–Menten equation (Eq. (2)).

$$-\frac{d[S]}{dt} = \frac{V_{max}[S]}{K_m + [S]} \quad (2)$$

For the analysis of progress curves a script was utilized applying non linear regression on the Lambert W solution of the Michaelis–Menten equation as it was published previously [15]. Progress curve analysis requires an estimation of K_m and V_{max}. This can be achieved by linearization of the integrated form of Michaelis–Menten equation, e.g. according to Eqs. (3) and (4) [14,17]. Estimates of K_m and V_{max} are calculated from the slope and intersection of y as shown in Fig. S4 (Supplementary data). Tables 1 and S2 summarize the results of the Lambert W function-analysis of progress curves.

$$\frac{t}{\ln([S]_0/[S])} = \frac{1}{V_{max}} \frac{[S]_0 - [S]}{\ln([S]_0/[S])} + \frac{K_M}{V_{max}} \quad (3)$$

$$\frac{t}{[S]_0 - [S]} = \frac{K_M}{V_{max}} \frac{\ln([S]_0/[S])}{[S]_0 - [S]} + \frac{1}{V_{max}} \quad (4)$$

2.8. Analysis of (+)-germacrene D synthase reaction

The Progress curve for the transformation of FDP was fitted in MATLAB 7.10.0.499 (R2010a) (MathWorks, Inc., Germany). For the analysis a script was utilized applying non linear regression on the Lambert W solution of the Michaelis-Menten equation [15]. The transformation of FDP is not complete (cf. main text, Fig. 6). The amount of residual unreacted FDP was calculated from the average of the last twelve data points (starting at 27075 s) to yield $[S]_{y0} = 34.9$ μM. Because Eq. (1) supposes complete transformation of the substrate, $[S]_{y0}$ was added to Eq. (1) and subtracted from the initial concentration $[S]_0$ to yield Eq. (5).

$$[S] = \left[K'_M W \left\{ \frac{[S]_0 - [S]_{y0}}{K'_M} \exp\left(\frac{\left([S]_0 - [S]_{y0}\right) - V'_{max} t}{K'_M} \right) \right\} \right] + [S]_{y0} \quad (5)$$

3. Results and discussion

The progress of the invertase reaction was monitored for different initial concentrations of sucrose. Spectra were acquired every 2 min over a period of several hours. Each spectrum was acquired with eight scans and a total acquisition time of 35 s. From each progress curve the initial rate was determined for a classical Michaelis–Menten analysis. Additionally, each progress curve was analyzed according to Eq. (1). The initial concentrations were chosen to be below, close to and above the literature value for K_m of 25 mM of sucrose [18].

Table 1
Kinetic parameters derived by progress curve analysis at different initial sucrose concentrations.

c_0 (sucrose) [mM]	K_m [mM]	v_{max} [μM/s]
3.50	15.5 ± 2.1	8.69 ± 1.1
8.70	26.3 ± 1.5	13.1 ± 0.6
12.4	26.9 ± 1.3	12.9 ± 0.5
14.8	28.2 ± 0.7	13.2 ± 0.3
15.1	30.0 ± 2.3	13.4 ± 0.8
17.8	26.8 ± 0.7	13.0 ± 0.2
22.3	26.7 ± 0.6	12.0 ± 0.2
27.4	29.2 ± 0.3	12.1 ± 0.1
41.8	38.8 ± 2.0	13.5 ± 0.4

In order to start the experiment sucrose has to be added to the invertase solution, the sample has to be inserted into the magnet and the magnet eventually has to be shimmed. The spectrometer was prepared prior to the experiment with an enzyme solution (tuning, matching and shimming). Addition of sucrose stock solution to a prepared invertase solution in an NMR tube did not yield a homogeneous mixture. Therefore both solutions were thoroughly mixed before they were filled into the NMR tube.

Fig. 1 shows all progress curves measured for the hydrolysis of sucrose. Each kinetic experiment was analyzed with respect to the concentrations of sucrose (H-1 of the glucose residue). In order to obtain the integral I_0 of sucrose at the beginning of each conversion ($t = 0$ s), I_0 was calculated from the sum of the integrals I_1 of sucrose (H-1 of glucose residue) and I_1 of the anomeric proton of α-glucose in the first spectrum. After the calculation of the concentrations, linear regression was applied to the first four data points of each progress curve (Fig. S1, Supplementary data). The slope directly yields the initial velocity for each experiment (Table S1, Supplementary data). The exact concentrations of all substrates and products were calculated using the peak of the acetate methyl group (buffer) as an internal reference (Fig. S2, Supplementary data). In addition, the time for starting the experiment was calculated from the linear equation by satisfying the constraint that the intersection of y is the same as the initial concentration $[S]_0$. In order to account for saturation effects, proton spectra of a mixture of sucrose, glucose and fructose were acquired applying the same conditions that were used for kinetic measurements (buffer, temperature, spectral parameters) but with increasing relaxation delays between 1 and 45 s. Each signal that was analyzed was corrected by a constant factor derived from the two proton spectra applying relaxation delays of 1 and 45 s (Fig. S3, Supplementary data).

Using NMR a complete analysis of the substrate and the products of sucrose cleavage is possible (see also Fig. S2, Supplementary data). This is demonstrated in Fig. 2 for an initial concentration of sucrose of 27.4 mM. The anomeric protons of free α- and β-glucose (H-1), H-1 of free β-fructofuranose and H-5 of β-fructopyranose were used for integration. Signals of the minor product α-fructofuranose could not be assigned unambiguously. The stereochemistry for the hydrolysis of sucrose by invertase has been studied previously [19–21]. Invertase is a ß-fructofuranosidase, hence the glycosidic bond is cleaved on the fructose residue. In agreement with the ß-fructofuranoside activity α-glucose is formed directly in the reaction, β-glucose only by mutarotation as is evident from Fig. 2A. Fig. 2B also reveals that the hydrolysis works under retention of the configuration of the β-D-fructofuranosyl residue as it was reported previously [21,22]. The formation of β-D-fructopyranose by mutarotation is evident from the time delay in its

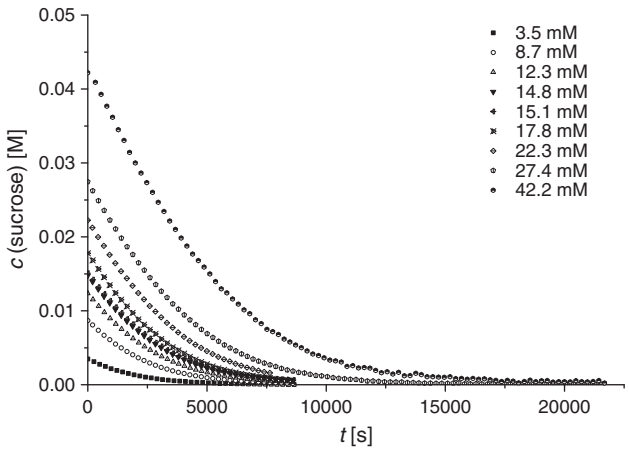

Fig. 1. Overview of progress curves recorded for the hydrolysis of sucrose by invertase. Initial concentrations of sucrose are listed in the legend (every second data point is shown).

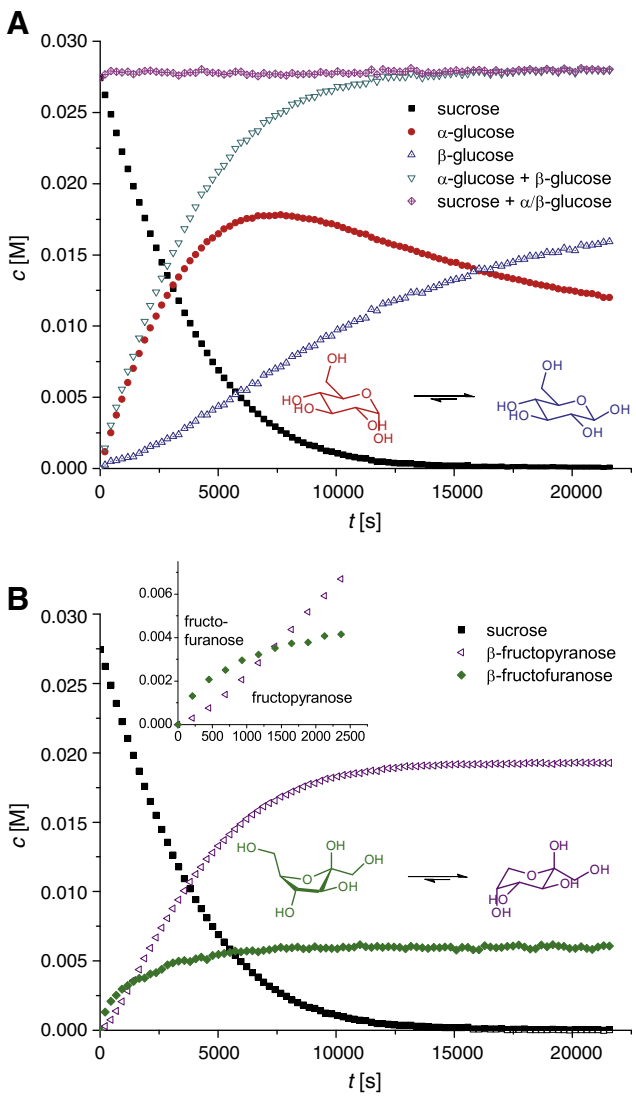

Fig. 2. NMR spectroscopic characterization of sucrose (27.4 mM) hydrolysis by invertase (every second data point is shown). A: Analysis of glucose formation; the reaction directly yields α-glucose, β-glucose is formed by mutarotation. The sum of total glucose and sucrose concentration was plotted as a control for signal stability over time. B: Analysis of fructose formation; cleavage of the glycosidic bond occurs under retention of the configuration of the anomeric center. Thus, the reaction directly yields β-fructofuranose, β-fructopyranose is also formed by mutarotation as indicated by the lag phase at the beginning of the curve.

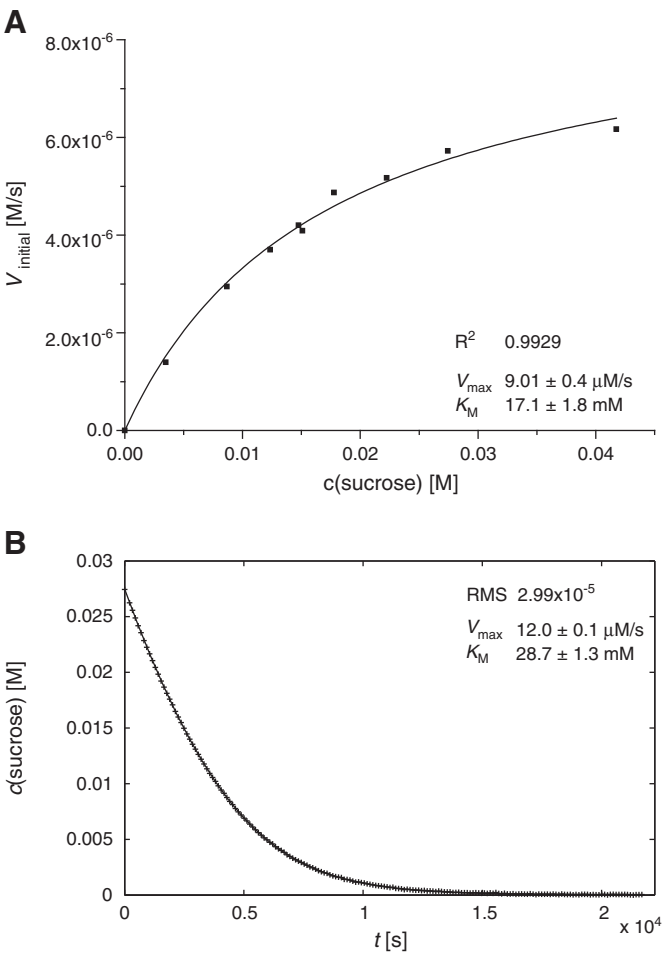

Fig. 3. Determination of K_m and V_{max}. A: Michaelis–Menten plot of invertase reaction derived from initial velocities of each progress curve. The fit afforded $K_m = 17$ mM and $V_{max} = 9$ µM/s. B: Progress curve analysis of invertase reaction with initial sucrose concentration of 27.4 mM. The data was fitted according to Eq. (1) and yielded $K_m = 29$ mM and $V_{max} = 12$ µM/s.

formation. Thus, all stereochemical information about the enzymatic reaction is obtained from a single experiment.

For comparison a Michaelis–Menten plot was derived from the NMR data (Fig. 3A, Table S1 in the Supplementary data). Initial rates were obtained by linear regression of the first four data points of each reaction and plotted against initial substrate concentration. The data was fitted directly to the Michaelis–Menten equation and afforded a K_m of 17 mM and a V_{max} of 9 µM/s (133 u/mg invertase). The K_m obtained by this method is slightly below the literature value of 25 mM and the specific activity is slightly below the supplier's specification of 200–300 u/mg invertase.

Fig. 3B shows the experimental data for an initial concentration of 27.4 mM sucrose and the progress curve analysis applying Eq. (1). Fitting of the data requires an estimation of K_m and V_{max} that can be derived by a linearization of the integrated form of Michaelis-Menten equation (see experimental section and Fig. S4). The fit afforded a K_m of 29 mM and a V_{max} of 12 µM/s (179 u/mg invertase). The quality of the fit is self-evident and the RMS is $3\ 10^{-5}$. For other initial concentrations between 8.7 and 22.3 mM we determined $K_m = 28.2 \pm 1.8$ mM and $V_{max} = 12.7 \pm 0.7$ µM/s (185 ± 15 u/mg Invertase). The results of all fits are summarized in Table 1. Noticeable K_m differs at the highest and lowest concentrations applied in the assay. For an initial concentration of 3.5 mM sucrose values obtained for K_m and V_{max} are 15.5 mM and 8.68 µM/s (128 u/mg sucrose), respectively. This indicates that substrate concentrations should not be too far away from the K_m as has been reported earlier for the analysis of progress curves [10]. For an initial concentration of 42 mM sucrose an increase in K_m is observed but V_{max} is still in the range of all other fits. This assumes competitive inhibition of the enzyme reaction, which could be originated from product inhibition. The errors quoted in table 1 reflect the quality of the fit and do not include systematic errors. These would become evident by running the time course repeatedly.

In order to investigate the effect of product inhibition, the reaction of invertase was repeated with 27 mM sucrose in the presence of a 1:1 mixture of glucose and fructose (26 mM). Fitting the progress curve according to Eq. (1) yielded $K_m = 38$ mM and $V_{max} = 12$ µM/s (Figs. 4 and S5 in the Supplementary data). The experiment demonstrates that product inhibition can be clearly seen already from two progress curves. Product inhibition has been known to be present in the action of invertase [23]. Fructose has been reported to be a competitive inhibitor and should result as observed here in an increase of K_m. Glucose is reported to reduce V_{max} as a (partial) non-competitive inhibitor.

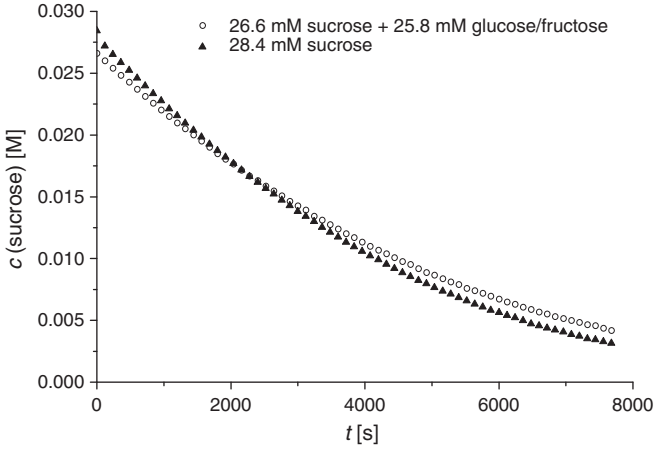

Fig. 4. Analysis of product inhibition. Comparison of progress curves of samples with 26 mM of fructose and glucose (1:1) added (○) and without addition of the products (▲) to the reaction of invertase. Addition of glucose and fructose clearly reduces the rate of the cleavage of sucrose. Fitting the progress curve of the reaction with added products according to Eq. (1) yielded expectedly a higher value for $K_m = 38$ mM but the same $V_{max} = 12$ μM/s. The increase in K_m is attributed to competitive product binding. Fitting the reaction progress without additional inhibition afforded $K_m = 28$ mM and $V_{max} = 11$ μM/s.

The inhibition constant K_i for glucose ($K_i \approx 0.4$ M) is much larger than that for fructose ($K_i \approx 0.2$ M) and thus the effect on V_{max} is not observable at the concentrations used here. The effect of the added fructose is however clearly discernable. In agreement with the observed product inhibition, progress curve analysis for an initial concentration of 42 mM sucrose led to an increase of K_m to 38.8 mM. In fact, in this experiment we have the product concentration at comparable levels already after about 60% of reaction progress.

In a further investigation we applied ^{1}H NMR spectroscopy to study the reaction of (+)-germacrene D synthase, (+)-GDS, from *S. canadensis*. The enzyme catalyzes the transformation of farnesyl diphosphate (FDP) to (+)-germacrene D, a monocyclic sesquiterpene that originates in the early steps of sesquiterpene biosynthesis. The enzyme was characterized previously by Prosser et al. [16]. They studied the Michaelis–Menten kinetics by a radioactive assay and obtained a K_m of 3 μM and a k_{cat} of 0.02 s^{-1}. The transformation of FDP starts with the cleavage of the diphosphate group under formation of a highly reactive carbenium ion. After cyclization, two hydride shifts and proton abstraction (+)-germacrene D is formed. The enzyme requires magnesium and/or manganese ions in the millimolar range to support the binding of FDP and the departure of the diphosphate group. The reaction catalyzed by (+)-germacrene D synthase is a single substrate reaction.

We were interested in the interaction of FDP with the enzyme as this type of interaction is also important in the posttranslational farnesylation of several regulative proteins by protein farnesyltransferase. FDP binding to a protein is driven by ionic as well as hydrophobic interactions due its amphiphilic nature. These types of interactions are very difficult to investigate and they normally require a very careful analysis of the state that the amphiphilic substrate is in. Often one finds heterogeneous solutions that have a drastic effect on the binding events. These consist of specific and possible unspecific hydrophobic binding to the protein as well as micelle formation of the substrate. We were able to characterize the model system (+)-germacrene D synthase in detail, making possible the characterization of FDP binding to the enzyme.

The analysis of the progress curve was only possible under specific conditions. 5% DMSO-d_6 was added to the NMR buffer (deuterated TBS) to keep the product (+)-germacrene D of the reaction in solution and to reduce micelle formation of FDP. To keep the protein native over the course of the reaction a reduced temperature of 285 K was used. We recorded spectra of 128 scans at intervals of 22 min over a period of 12 h. The signals of H-2 [5.47 ppm (*t*, 1H, $J = 7.0$ Hz)] of FDP and H-5 [5.76 ppm (*d*, 1H, $J = 15.9$ Hz)] of (+)-germacrene D were used for the kinetic analysis in the spectra of FDP transformation. Saturation effects were neglected ($T_1 < 1.5$ s for both protons). In Fig. 5A the integrals of both protons as well as their sum are plotted against reaction time. The first data point was set to 13 min. This takes into account 2 min after addition of FDP to (+)-GDS for the submission of the sample to the magnet, locking, shimming and starting the experiment. Additionally, the spectrum represents the time averaged signal over a period of 22 min. Therefore the time for each data point is set to the middle of this period. The curve of FDP does not show typical exponential behavior and is reduced compared to formation of (+)-germacrene D. The reduction of signal intensities is attributed to micelle formation. For FDP in aqueous solution a CMC of 12 mM is reported but we have observed that micelle formation of FDP is promoted by magnesium ions even at micromolar concentrations of Mg^{2+} [24]. Due to micelle formation signal intensities of FDP are reduced even in presence of DMSO. Under the reaction conditions, 500 μM FDP and 1 mM $MgCl_2$, the signal intensities are reduced by 45% as a result of line broadening (Fig. S6, Supplementary

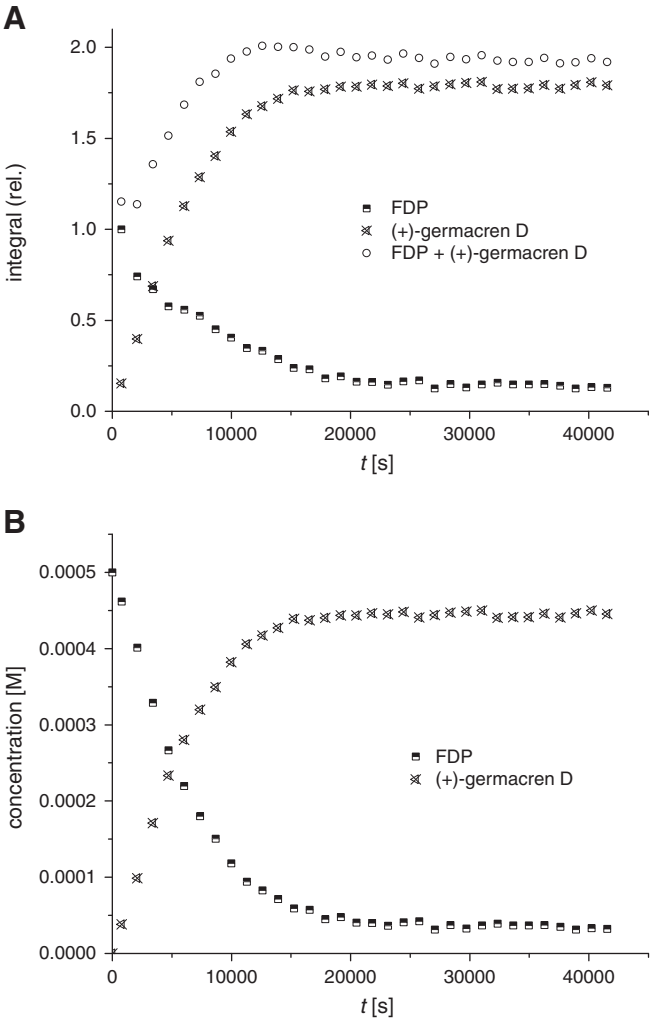

Fig. 5. Progress curves for the reaction of farnesyl diphosphate (FDP) with germacrene D synthase to yield (+)-germacrene D. A: While the product formation shows typical exponential kinetics, this behavior is not observed for FDP. The sum of both curves is not constant over time. This observation is explained by micelle formation of FDP in presence of $MgCl_2$. B: In order to obtain the progress curve for the transformation of FDP, the integrals in Fig. 5A were corrected as described. Calculating the concentrations and plotting the concentration time course for FDP and (+)-germacrene D now shows two curves that intersect at half of the concentration for both compounds.

data). The effect of signal reduction is concentration dependent as can be seen in Fig. 5A. When the concentration of FDP and hence of the micelles is reduced the signal reduction decreases. This can be observed from the sum of integrals that increases with the progress of the reaction. In order to account for this effect, all integrals of FDP are corrected, assuming that the sum of integrals for FDP and (+)-germacrene D from minute 13 to 211 have a constant value. In Fig. 5B the progress curves for FDP and germacrene D are plotted after applying the correction and calculating the concentrations.

Fig. 6 shows the progress curve derived under the conditions of the NMR assay. The kinetic parameters obtained are $K_m = 379\ \mu M$ and $k_{cat} = 0.04\ s^{-1}$. k_{cat} is in the same range of the value obtained by Prosser et al., but there is a 125-fold increase in K_m. To some extend this increase is explained by the change of reaction conditions. The radioactive assay was conducted in a MOPSO buffer that contained 5 mM $MgCl_2$ and was overlaid with hexane to capture (+)-germacrene D from the aqueous phase and the reaction temperature was 303 K [16]. In addition to that we observe product inhibition by (+)-germacrene D. The enzymatic transformation yielded a clear reaction solution and gave no indication for the formation of an emulsion. A strong association tendency of (+)-germacrene D to the hydrophobic active site in this aqueous environment is reasonable. This conclusion is supported by the fact that approximately 6% of the substrate is not transformed even after 12 h. Due to the energetics of the reaction the backward reaction can be excluded and the incomplete transformation is thus not attributed to an equilibrium being present. Taking these effects into account, the increase of K_m is reasonable.

4. Conclusions

We demonstrated here that NMR spectroscopy is a powerful tool to analyze the kinetics of enzyme reactions using progress curves. NMR spectroscopy is one of many methods that can be applied in substrate or product detection. Experiments in enzyme kinetics have to be planed carefully and it is very important to choose the adequate technique. NMR spectroscopy, however, can easily detect and quantify in situ all reaction products and substrates. Also, stereochemistry of the products formed is directly obtained from the spectra which other methods cannot normally achieve. Obviously NMR is not the method to characterize very fast enzyme reactions but many transformations are eligible when the reaction conditions are adapted carefully. The detection of product inhibition is possible by comparing the time courses in only two experiments. The protocol presented here is also a simple and direct approach for the measurement of enzyme kinetics in the presence of synthetic inhibitors. The closed shell solution of the Michaelis–Menten equation can be expanded to further kinetic models including various types of inhibition models [14]. In routine applications it is not necessary to run experiments for as much time as documented here. The experiments can also be run with as little as ten data points. Progress curve analysis of the change in substrate concentrations as a function of time requires only a very straightforward and simple experimental setup to determine enzyme parameters with a minimal number of experiments.

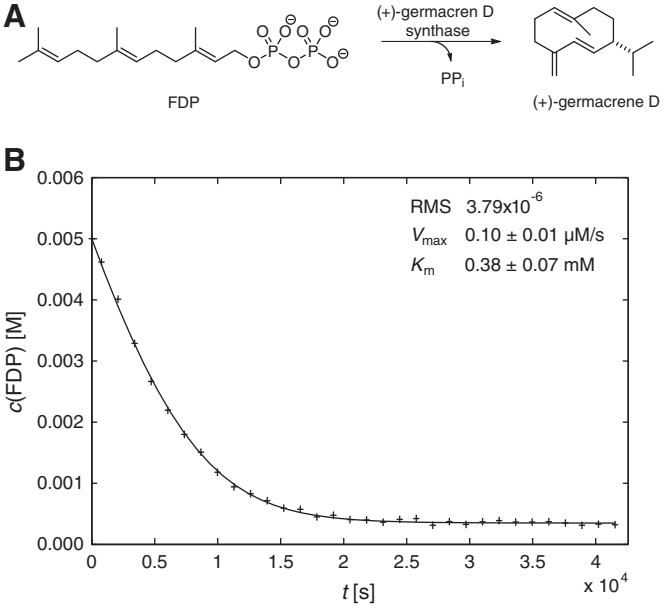

Fig. 6. Progress curve analysis of farnesyl diphosphate (FDP) transformation to the hydrocarbon (+)-germacrene D A: Scheme for the reaction catalyzed by (+)-germacrene D synthase. B: Progress curve for the transformation of 500 µM FDP by (+)-GDS (2.5 µM) in deuterated 0.05 M TBS (5% DMSO-d_6) and 1 mM $MgCl_2$. The transformation was not complete (6% unreacted because of product inhibition). This was accounted for by a constant term implemented in Eq. (1) (cf. Eq. (5)). The fit afforded $K_m = 379\ \mu M$ and $V_{max} = 0.10\ \mu M/s$ ($k_{cat} = 0.04\ s^{-1}$).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbapap.2011.10.011.

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