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The use of reaction timecourses to determine the level of minor contaminants in enzyme preparations

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ABSTRACT

Enzyme mutagenesis is a commonly used tool to investigate the structure and activity of enzymes. However, even minute contamination of a weakly active mutant enzyme by a considerably more active wild-type enzyme can partially or completely obscure the activity of the mutant enzyme. In this work, we propose a theoretical approach using reaction timecourses and initial velocity measurements to determine the actual contamination level of an undesired wild-type enzyme. To test this method, we applied it to a batch of the Q215A/R235A double mutant of orotidine 5′-monophosphate decarboxylase (OMPDC) from *Saccharomyces cerevisiae* that was inadvertently contaminated by the more active wild-type OMPDC from *Escherichia coli*. The enzyme preparation showed significant deviations from the expected kinetic behavior at contamination levels as low as 0.093 mol%. We then confirmed the origin of the unexpected kinetic behavior by deliberately contaminating a sample of the mutant OMPDC from yeast that was known to be pure, with 0.015% wild-type OMPDC from *E. coli* and reproducing the same hybrid kinetic behavior.

Enzyme kinetics is an important tool in understanding how enzymes are able to so successfully catalyze an incredibly broad range of chemical reactions. Knowing the fundamental kinetic parameters associated with both the overall enzymatic reaction and the individual mechanistic steps of the reaction can aid in the knowledge of how the enzyme is able to effect such efficient catalysis. The steady-state kinetic parameters are usually determined using Michaelis–Menten kinetics [1]. Eq. (1) is the Michaelis–Menten equation. In the equation, v is the observed initial velocity of the reaction. It is equivalent to either the initial rate of product formation, $d[P]/dt$, or the initial rate of substrate consumption, $-d[S]/dt$. [E] and [S] give the concentration of enzyme and substrate, respectively. k_{cat} is a first-order rate constant that represents the maximal turnover number for the enzyme. K_m, also known as the Michaelis constant, is the substrate concentration at which the enzyme is forming product at half of its maximal rate. It also gives an idea of how much substrate is needed in order to saturate the enzyme given that the enzyme will be saturated when [S] >> K_m. The ratio of the parameters, k_{cat}/K_m, gives a second-order rate constant that can be used as a measure of an enzyme's overall efficiency in carrying out a particular catalysis. The usual experimental approach is to measure the initial velocity of either product formation or substrate consumption for several substrate concentrations and fit these data to Eq. (1) to provide the kinetic parameters k_{cat} and K_m:

$$v = \frac{d[P]}{dt} = -\frac{d[S]}{dt} = \frac{k_{cat}[E][S]}{K_m + [S]} \quad (1)$$

Under conditions where substrate concentration is less than approximately 10% of K_m, an approximation is made that the [S] term in the denominator of Eq. (1) can be ignored, reducing the Michaelis–Menten equation to Eq. (2). Of note is that the initial velocity of the reaction is now directly proportional to substrate concentration, resulting in a first-order decay of the substrate. The observed first-order rate constant, k_{obs}, is related to k_{cat}/K_m by Eq. (3). Thus, by plotting a timecourse of reaction progress at sufficiently low substrate concentration, the second-order rate constant, k_{cat}/K_m, can be determined directly. However, this approach does not allow for the determination of k_{cat} or K_m individually. The two experimental approaches of measuring initial velocities at many substrate concentrations and observing the timecourse of reaction progress at low initial substrate concentrations provide complementary techniques of determining k_{cat}/K_m for an enzyme. Ideally, both would be used so that a timecourse of reaction progress could be used as an internal check of k_{cat}/K_m for the enzymatic catalysis determined from initial velocity experiments:

$$v = \frac{k_{cat}[E][S]}{K_m} \quad (2)$$

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$$k_{obs} = \frac{k_{cat}}{K_m}[E]. \quad (3)$$

However, if the enzyme of interest has been contaminated with another more active enzyme that catalyzes the same chemical reaction, the kinetics could become more complicated. This can commonly be seen when the enzymes are prepared by overexpressing them from *Escherichia coli* that contains its own version of the enzyme. A wild-type enzyme obscuring the reactivity of a mutant enzyme with lower activity has been reported previously for several enzymes, including triosephosphate isomerase [2], β-galactosidase [3], and UDP-*N*-acetylglucosamine enolpyruvyl transferase [4]. In these cases, the mutants of the studied enzymes were so inactive toward catalysis that small amounts of highly active wild-type enzymes were responsible for the entirety of the observed enzymatic activity. The kinetic parameters that were observed in these cases were inconsistent with other kinetic measurements or the chemical knowledge of what effect the mutations should have, for instance, predicting that mutating critical residues in the active site would have no effect on the rate of catalysis. The primary difference compared with the experimental results described in this work is that our contaminating enzyme contributed some, *but not all,* of the observed catalytic activity.

Materials and methods

Materials

Orotidine 5′-monophosphate (OMP)[2] was available from our earlier studies [5–8]. Water was obtained from a Milli-Q Academic purification system. All other chemicals were reagent grade or better and were used without further purification. Wild-type orotidine 5′-monophosphate decarboxylases from *Saccharomyces cerevisiae* (ScOMPDC) and *E. coli* (EcOMPDC) were available from earlier studies [5,8,9]. The protein sequence of ScOMPDC differs from the published sequence for wild-type yeast OMPDC by the following mutations: S2H, C155S, A160S, and N267D [5,9]. Except for the C155S mutation, the sequence is the same as that observed in the published crystal structure of wild-type ScOMPDC. The C155S mutation increases the stability of the protein but does not affect the kinetic parameters or the overall structure of the enzyme [10]. The batch of ScOMPDC containing the Q215A/R235A double mutation was prepared according to the method given in our earlier studies [5,8,9], although the batch prepared for the current work was shown to be contaminated by approximately 0.09 mol% of wild-type EcOMPDC from the *E. coli* host used for overexpression of the mutant yeast enzyme (vide infra).

Preparation of solutions

Solution pH was determined at 25 °C using an Orion model 720A pH meter equipped with a Radiometer pHC4006-9 combination electrode that was standardized at pH 7.0 and 10.0 at 25 °C. Stock solutions of OMP were adjusted to pH 6.0–7.0 and stored in small aliquots at −20 °C. The concentration of OMP in stock solutions was determined from its absorbance in 0.1 M HCl at 267 nm using $\varepsilon = 9430\ M^{-1}\ cm^{-1}$ [11].

Determination of k_{cat}, K_m, and k_{cat}/K_m for decarboxylation of OMP

Samples of mutant yeast OMPDCs that had been stored at −80 °C were defrosted and extensively dialyzed at 4 °C with 5 mM MOPS (50% free base) at pH 7.1 and $I = 0.05$ (NaCl). The concentration of OMPDC in the stock solution was determined from its absorbance at 280 nm using an extinction coefficient of $29{,}910\ M^{-1}\ cm^{-1}$, calculated using the ProtParam tool available on the ExPASy server [12].

Second-order rate constants k_{cat}/K_m ($M^{-1}\ s^{-1}$) for decarboxylation of OMP catalyzed by OMPDC were determined in 10 to 30 mM MOPS (50% free base) at pH 7.1, 25 °C, and $I = 0.105$ (NaCl). Initial velocities of decarboxylation of OMP, v_o ($M^{-1}\ s^{-1}$), were determined spectrophotometrically by following the decrease in absorbance at 279 nm ($\Delta\varepsilon = -2400\ M^{-1}\ cm^{-1}$), 290 nm ($\Delta\varepsilon = -1620\ M^{-1}\ cm^{-1}$), or 295 nm ($\Delta\varepsilon = -842\ M^{-1}\ cm^{-1}$) [5,6]. The value of k_{cat}/K_m was obtained from the observed first-order rate constant, k_{obs} (s^{-1}), for the complete reaction of OMP at $[OMP]_o << K_m$, monitored spectrophotometrically at 279 or 290 nm, using the relationship $k_{cat}/K_m = k_{obs}/[E]$ (Eq. (3)). Values of k_{cat} and K_m were obtained individually from a Michaelis–Menten plot of initial velocity versus [OMP] with initial OMP concentrations ranging from 100 to 2000 μM, monitored spectrophotometrically at 279, 290, or 295 nm using the relationship $v = k_{cat}[E][OMP] / (K_m + [OMP])$ (Eq. (1)).

Theory

We simulated the behavior of a mixture of a large amount of poorly active mutant enzyme and a much smaller amount of highly active wild-type enzyme. If the assumption is made that in a mixture of enzymes each enzyme will behave independently (as opposed to cross-dimerization or some other interaction), then the overall initial velocity of reaction will be the sum of the initial velocities for each enzyme (Eq. (4)). The initial velocities of reaction contributed by wild-type and mutant enzymes, v^{wt} and v^{mut}, will be given by the Michaelis–Menten expression (Eq. (1)), corrected for the fraction of either wild-type or mutant enzyme present, f^{wt} or f^{mut}. If the wild-type enzyme is present in trace amounts, the approximation is made that $f^{mut} = 1$. Eq. (5) is derived by taking the absolute velocity of reaction due to mutant enzyme at any substrate concentration and scaling it by dividing by the maximum possible velocity for this mixture of wild-type and mutant enzymes, $k_{cat}{}^{wt}f^{wt}[E] + k_{cat}{}^{mut}[E]$. The analogous equation for the scaled velocity of reaction due to the wild-type enzyme is not shown. Eq. (6) gives the fractional velocity of reaction due to the mutant enzyme, by taking the velocity of reaction catalyzed by mutant enzyme at any given substrate concentration and dividing by the total observed velocity of reaction catalyzed by the mixture of the two enzymes at that specific substrate concentration. Again, the analogous equation for the fractional velocity of reaction by wild-type enzyme is not shown. Note that whereas the absolute velocity of reaction will depend on the total enzyme concentration, Eqs. (5) and (6) define ratios of velocities and are not dependent on the enzyme concentration:

$$v = v^{wt} + v^{mut} = f^{wt}\frac{k_{cat}^{wt}[S][E]_{total}}{K_m^{wt} + [S]} + f^{mut}\frac{k_{cat}^{mut}[S][E]_{total}}{K_m^{mut} + [S]} \quad (4)$$

$$v_{scaled}^{mut} = \frac{v^{mut}}{v_{max}^{mut} + v_{max}^{wt}} = \frac{\left(\frac{k_{cat}^{mut}[S]}{K_m^{mut}+[S]}\right)}{k_{cat}^{mut} + k_{cat}^{wt}f^{wt}} \quad (5)$$

$$v_{fractional}^{mut} = \frac{v_{mut}}{v^{mut} + v^{wt}} = \frac{k_{cat}^{mut}}{k_{cat}^{mut} + k_{cat}^{wt}f^{wt}\left(\frac{K_m^{mut}+[S]}{K_m^{wt}+[S]}\right)}. \quad (6)$$

A mixture of wild-type and mutant enzymes can be divided into three classes. In class 1, the contamination is so minor, or the mutant enzyme is sufficiently active, that the observed activity

[2] *Abbreviations used:* OMPDC, orotidine 5′-monophosphate decarboxylase; OMP, orotidine 5′-monophosphate; ScOMPDC, orotidine 5′-monophosphate decarboxylase from *Saccharomyces cerevisiae*; EcOMPDC, orotidine 5′-monophosphate decarboxylase from *Escherichia coli*; UMP, uridine 5′-monophosphate; MOPS, 3-(N-morpholino)propanesulfonic acid.