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Single time-point analysis of product and substrate inhibition

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When enzyme inhibition by either the product or excess substrate occurs, it is possible to determine the characteristic kinetic parameters based on [P]/*t* measurements, even when a large proportion of the substrate is converted. The advantages of various approaches are discussed. Most of them allow a good estimation of the *V* and K_m values. Conversely, the determination of K_p (product inhibition) and K_i (inhibition by excess substrate) can be more challenging. In the first case, determination of the type of inhibition requires more complex experiments that are beyond the scope of the present contribution. In the second, the inhibition constant K_i can only be roughly estimated. In an experimental approach, we compared the results obtained either with initial rate measurements or with 50 to 60% conversion of the substrate. Similar values of *V* and K_m were obtained. Measurements involving the conversion of a large proportion of substrate are particularly advantageous when the assay method is difficult or time-consuming, or when obtaining the substrate presents experimental difficulties or involves substantial costs.

Keywords Inhibition by substrate, Inhibition by product, Time-point, Initial rate, Enzyme kinetic parameters

Abbreviations

S	Substrate
P	Product
E	Enzyme
v	Initial rate
HMM	Henri-Michaelis–Menten
HW	Hanes-Woolf
SD	Standard deviation
SE	Standard error

In a previous study[1], we have shown that, if some simple conditions are fulfilled, it is still possible to accurately derive the steady-state kinetics parameters *V* and K_m from single time-point measurements even at high levels of substrate (S) conversion. Table S1 summarises the systematic errors that prevail when the [P]/*t* ratio is substituted for the true initial rate (*v*) in the Henri-Michaelis–Menten equation (HMM, Eq. 1) or its linearised Hanes-Woolf (HW) form (Eq. 1b).

$$v = \frac{V \cdot [S]_0}{K_m + [S]_0} \tag{1}$$

$$\frac{[S]_0}{v} = \frac{K_m + [S]_0}{V} \tag{1b}$$

Note that utilisation of the integrated HMM equation (Eq. 1c, where [P] is the product concentration at time *t*) directly yielded good values of both parameters.

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$$t = \frac{[P]}{V} + \frac{K_m}{V} \cdot \ln\left(\frac{[S]_0}{[S]_0 - [P]}\right) \tag{1c}$$

One of the boundary conditions that was used in this initial study was the absence of inhibition by S or P. In the present paper, we analyse situations where these phenomena occur, and propose the easiest solutions to derive the characteristic kinetic parameters. One of our goals is to reduce the number of measurements to a minimum when, for instance the assay is very time-consuming, or the substrate is expensive or difficult to obtain.

As before, some important conditions must be fulfilled.

1. For sufficiently long incubation times, the reaction is complete or nearly complete for the considered substrate. Note that a reversible reaction can be made irreversible by removing one of the products. In the case of a multi-substrate system, the concentration(s) of the other substrate(s) is (are) such that it (they) can be considered as constant.
2. The enzyme does not lose activity during the incubation period. This can be easily verified with the help of Selwyn's test[2].
3. There is no non-enzymatic disappearance of S.
4. There is no evidence of hysteresis behaviour[3] (burst or lag).
5. The [P]/t *vs.* $[S]_0$ curve does not indicate a cooperative behaviour.

Note that the corresponding controls are also necessary when the initial rate method is utilised.

Competitive inhibition by the product

This phenomenon implies the binding of the product to the free enzyme with the formation of an EP complex, characterized by a dissociation constant K_p.

If the inhibition is competitive and there is no product at $t = 0$, the time-course of the reaction obeys Eq. (2)[4]

$$V \times t = \left(1 - \frac{K_m}{K_p}\right) \cdot [P] + K_m \cdot \left(1 + \frac{[S]_0}{K_p}\right) \cdot \ln\left(\frac{[S]_0}{[S]_0 - [P]}\right) \tag{2}$$

that can be rearranged as

$$\frac{[P]}{t} = \frac{V}{1 - \frac{K_m}{K_p}} - \frac{K_m}{t} \cdot \frac{K_p + [S]_0}{K_p - K_m} \cdot \ln\left(\frac{[S]_0}{[S]_0 - [P]}\right) \tag{3}$$

When some product is added together with the substrate at t = 0, Eq. (4) applies[4]:

$$V \times t = \left(1 - \frac{K_m}{K_p}\right) \cdot ([P] - [P]_0) + K_m \cdot \left(\left(1 + \frac{[S]_0 + [P]_0}{K_p}\right) \cdot \ln\left(\frac{[S]_0}{[S]_0 + [P]_0 - [P]}\right)\right) \tag{4}$$

Inhibition by the substrate

Our analysis rests on the simple model:

$$\begin{array}{ccc} E + S \rightleftharpoons & ES & \rightarrow E + P \\ & \downarrow\uparrow & K_i = \frac{[ES].[S]}{[ES_2]} \\ & ES_2 & \end{array}$$

Equation 5 and 6 are respectively the initial rate and integrated equations[4].

$$v = \frac{V \cdot [S]_0}{[S]_0 + K_m + \frac{[S]_0{}^2}{K_i}} \tag{5}$$

$$V \cdot t = [P] + \frac{[S]_0{}^2 - [S]^2}{2K_i} + K_m \cdot \ln\left(\frac{[S]_0}{[S]}\right) \tag{6}$$

Results

Theory and simulations

Competitive inhibition by the product

Product inhibition can easily be detected by adding product (P) together with the substrate. If initial rates are measured without adding P, the V and K_m values are not influenced by P under these conditions. However, this does not allow to determine K_p, the dissociation constant of the EP complex. In theory, it is possible to obtain the K_p value by continuously monitoring [S] or [P] *vs.* t as shown by Eqs. (2) and (3)[4]. However, is it also possible to derive the 3 parameters (V, K_m, K_p) from single time-points as done in Ref.[1] for V and K_m in simple systems?

In a first approach and with the help of Eq. (3), we simulated the time needed to reach 10, 30 or 60% of substrate conversion at the lowest substrate concentration with K_p values of 0.125, 0.25, 0.5, 1.0, 2.0 and 4, and

K_m and V values of 1.0 as in Ref.[1]. Unless otherwise stated, 18 simulations were performed in each case in all the following analyses.

Not unexpectedly, in the absence of errors (4 significant digits) and by applying Eq. (3), Matlab retrieved the correct values for the 3 parameters.

When rounding to 2 significant digits, (maximum errors < 2%), and although the retrieved values of V (0.89–1.12, maximum SE 16%) and K_m (0.96–1.06, maximum SE 8%) were very good, the K_p values were much less satisfactory (see Table S2): four retrieved values were clearly strongly overestimated (9.5 *vs.* 0.5, 2300 *vs.* 2, 2800 *vs.* 4 and 77 *vs.* 4) and the SEs were always quite large, even when the retrieved K_p value was acceptable.

When errors up to 5 or 10% were introduced, the situation was much worse (see Tables S3 and S4). In many cases, absurd values were retrieved and even for the "reasonable" values, the SEs could be quite large. Therefore, it is highly risky to unquestionably rely on the results obtained using the integrated equation when even minor errors are detected, even if the results initially seem "reasonable".

In regard of these rather poor results, it became interesting to analyse the same data on the basis of the HMM equation (with the help of the HW linearisation, Eq. 1b) without taking account of the actual product inhibition. The outcome (SM and Table S5) was rather surprising (at least for us). In all cases, even with errors up to 10%, the V_{app} and $(K_m)_{app}$ values were reasonable, and the latter were in agreement with those recorded for the system without the occurrence of product inhibition (see Table S1). The V_{app} values were underestimated by a factor 2 at 60% substrate conversion with $K_m/K_p = 8$ but quite good in all other cases.

In consequence, it seems that reasonable values of $(K_m)_{app}$ and V_{app} can be derived by completely neglecting the product inhibition at least up to a K_m / K_p ratio of 8. Above this ratio, the measured V_{app} is expected to further decrease. Indeed, with $K_p = 0.0625$: $(K_m)_{app} = 1.61 \pm 0.34$ and $V_{app} = 0.38 \pm 0.04$ (no error), and with $K_p = 0.03$: $(K_m)_{app} = 1.59 \pm 0.38$ and $V_{app} = 0.23 \pm 0.03$. Although not very good, these results are certainly not catastrophic and can be very easily improved (see below).

Of course, direct application of the simple HMM Eq. (1) cannot yield a value of K_p and one can wonder if the K_p and better V and K_m values can be obtained by adding some product at the very beginning of the reaction. To study this possibility, we selected a K_p value of 1 and initial [P] ($[P]_0$) values of 0.75, 1.5, 2.25 and 3.0. Simulations were performed on the basis of Eq. (4) and when errors up to 10% were introduced (see SM), the analysis on the basis of the integrated equation Eq. (3) yielded very poor results.

On the other hand, the true initial rates obey Eq. (7) and allow to compute V, K_m and K_p:

$$v = \frac{V.[S]_0}{[S]_0 + K\prime_m} \tag{7}$$

Or

$$\frac{v}{[S]_0} = \frac{K\prime_m + [S]_0}{V} \tag{7b}$$

in the linear HW form where

$$K\prime_m = K_m \times \left(1 + \frac{[P]_0}{K_P}\right) \tag{8}$$

V and K'_m values are often derived from HW plots drawn for each $[P]_0$ value, and subsequently K_m and K_p values from K'_m *vs.* $[P]_0$ secondary plots. In a "no error" situation, the expected K_m and K_p values were (not unexpectedly) retrieved. However, when secondary plots are involved and errors introduced, the deduced parameters can be much less reliable. As shown in Table S7, with errors up to 5%, the V_{app} values resulting from the primary HW plots are quite good and the $(K_m)_{app}$ and K_p values deduced from the secondary plot differ from the real ones by factors of at most 3 and 5 for the K_m and K_p values, respectively, although large SEs are recorded. When errors are increased up to 10%, some results become absurd even with the real initial rates, and the SEs become enormous and sometimes even larger than the values themselves.

It is much more rigorous to fit all the data points directly to Eq. (9) ("Global Fitting", n−3 degrees of liberty for n measurements). To analyse this strategy, we performed fittings with 35, 15 or 12 data points. On the basis of Eq. (4) that takes account of the possible presence of P at $t = 0$, the exact values of t were adjusted to obtain 60% of substrate conversion at the lowest $[S]_0$ (0.35). The other $([P] - [P]_0)/t$ values were then calculated at the same t (constant time strategy) but to reach 60% conversion at the lowest $[S]_0$ in all cases, t had to be increased with the $[P]_0$ value (see legend of Table 1 for details).

Random errors up to 10% were then introduced on these exact data points and the corresponding $([P] - [P]_0)/t$ values calculated. Fitting was then performed according to Eq. (9) yielding V_{app}, $(K_m)_{app}$ and $(K_p)_{app}$ values (wrongly assuming that the $([P] - [P]_0)/t$ values corresponded to initial rates). This was repeated 20 times and the results are displayed in Table 1.

$$\frac{[P] - [P]_0}{t} = \frac{V_{app}.[S]_0}{[S]_0 + (K_m)_{app}.\left(1 + \frac{[P]_0}{(K_p)_{app}}\right)} \tag{9}$$

With 35 data points, the results are excellent. The ranges and the SEs are similar (note that the V_{app} and $(K_m)_{app}$ values should be compared to those of Table S1 for 60% conversion). With 15 and 12 data points, the ranges and SEs not unexpectedly increase. Note that with 12 points, one value of $(K_m)_{app}$ (out of 20 combinations) was > 3.0. It seems that 15 measurements represent a strict minimum if reasonable values are to be expected.

Strategy	V (Fixed K_p) or V_{app}				K_m (Fixed K_p) or $(K_m)_{app}$				K_p			
	Range	Average	Range SEs (%)	Average SE (%)	Range	Average	Range SEs (%)	Average SE (%)				
Poor Fixed K_p	0.62–0.97	0.76 ± 0.10	8–20	14	0.80–1.69	1.10 ± 0.28	16–38	27	Fixed $K_p = 1.5$, real $K_p = 0.25$ (poor guess)			
Good Fixed K_p	0.82–1.31	0.98 ± 0.11	6–17	12	0.64–1.53	0.96 ± 0.21	13–33	23	Fixed $K_p = 1.0$, real $K_p = 1.0$ (good guess)			
Poor Fixed K_p	0.98–1.49	1.19 ± 0.14	7–15	11	0.67–1.40	1.02 ± 0.22	12–26	19	Fixed $K_p = 0.66$, real $K_p = 4.0$ (poor guess)			
HW Eq. (1b)	0.67–0.95	0.78 ± 0.09	7–16	11	1.35–2.41	1.75 ± 0.37	12–29	22	$K_p = 0.25$ (not involved in the HW analysis)			
HW Eq. (1b)	0.84–1.27	1.01 ± 0.10	6–15	10	1.15–2.48	1.72 ± 0.33	12–28	19	$K_p = 1.0$ (not involved in the HW analysis)			
HW Eq. (1b)	0.98–1.42	1.15 ± 0.11	5–12	10	1.29–2.49	1.84 ± 0.33	11–23	18	$K_p = 4.0$ (not involved in the HW analysis)			
									$(K_p)_{app}$			
									Range	Average	Range SEs (%)	Average SE (%)
Global fitting												
35 points	0.84–1.25	1.02 ± 0.09	5–8	6	1.32–2.35	1.72 ± 0.24	10–15	12	1.02–1.29	1.13 ± 0.08	6–9	8
15 points	0.88–1.30	1.04 ± 0.12	4–13	8	1.33–2.57	1.82 ± 0.33	8–22	15	1.00–1.41	1.19 ± 0.12	6–16	13
12 points	0.82–1.60	1.11 ± 0.21	6–15	11	1.14–3.70	2.02 ± 0.66	10–30	20	0.95–1.58	1.19 ± 0.15	7–17	13

Table 1. Inhibition by P. Comparison between the HW linearisation, the "Fixed K_p" and the "Global fitting" strategies. In all cases, substrate conversion was 60% at the lowest $[S]_0$ (constant time strategy) and the errors up to 10%. For the "HW" and "Fixed K_p" strategies, the exact $[P]/t$ values were calculated on the basis of Eq. (3) (with $[P]_0 = 0$) that involves the real K_p value. With the "HW" strategy it was assumed that $[P]/t = v$ and the simulated data were analysed without taking account of the inhibition by P (Eq. 1b). The "Fixed K_p" strategy (details in Tables S10 and S11) utilised the same simulations but the fitting was performed with the integrated Eq. (3). Global fitting was done on 35, 15 or 12 points (32, 12 or 9 degrees of liberty, respectively). For 35 points, $[S]_0$ values were 0.35, 0.70, 1.05, 1.40 1.75, 2.10 and 2.45 and $[P]_0$ values 0, 0.75, 1.50, 2.25 and 3.0. For 15 points, $[S]_0$ values were 0.35, 0.70, 1.05, 1.75 and 2.45 and $[P]_0$ values 0, 0.75 and 1.5. For 12 points, $[S]_0$ values were 0.35, 1.05, 1.75 and 2.45 and $[P]_0$ values 0, 1.5 and 3.0. To reach 60% conversion at the lowest $[S]_0$, t had to be increased with the $[P]_0$ value (i.e. 1.237, 1.924, 2.11, 3.299 and 3.986 for $[P]_0 = 0$, 0.75, 1.5, 2.25 and 3.0, respectively). The V_{app} and $(K_m)_{app}$ values (HW and Global fitting) should be compared to those given in Table S1 for 60% of substrate conversion.

An alternative strategy would be to obtain an independent estimation of K_p, for instance by equilibrium dialysis, ITC, rapid filtration of a negligible fraction of the mixture through an adequate membrane or any other direct technique.

This approach reduces the number of parameters to be determined on the basis of the kinetic experiments from 3 to 2 which is a very clear advantage. To perform the analysis, we selected 2 different situations. In the first one ("good values"), the "fixed" K_p value was within 66 and 150% of the real one while in the second one, the "fixed" K_p was over or underestimated by a factor of 6 ("poor values"). No product was present at $t = 0$. The results are summarised in Table 1 and the details given in the SM (Tables S9 and S10). The conclusions are quite clear. In all cases, the derived V and K_m (integrated equation) or $(K_m)_{app}$ values were always rather close to the correct ones (within a factor of at most 1.8). The same results were analysed on the basis of the HW linear equation that does not take account of the inhibition by P and the results were even better than with the integrated equation if one takes account of the overevaluation of $(K_m)_{app}$ described by Table S1. In fact, the $(K_m)_{app}$ values were in the range of 67 to 140% of the expected ones, which can be considered as a very good result. Surprisingly, there was not much difference between the "good" and "poor" fixed K_p values. The SEs on the individual values were acceptable: with the integrated equation, they ranged from 1 to 20% for V and from 3 to 36% for K_m and they were quite similar with the HW linearisation: 1 to 16% for V_{app} and 3 to 28% for $(K_m)_{app}$. The values themselves deviate by a factor smaller than 2 even in the worst cases.

Table 1 compares the results obtained with the HW linear Eq. (1b) (that does not take account of the inhibition by P), with the "Fixed K_p" and "Global fitting" strategies. The results are rather similar and generally quite

good although V is somewhat underestimated with the "HW" and "Fixed K_p" strategies when the real K_p is low. Thus, if one wishes to reduce the number of data points to a minimum, the simple "HW" and the "fixed K_p" (that involves an independent estimation of K_p) strategies are probably the best choices. If additional experiments do not represent an important amount of work, the "Global Fitting" strategy (with for instance 15 measurements) represents a very useful complement. Indeed, a major difference between the directly measured value of K_p and that derived from the "Global Fitting" would suggest a "non-competitive" contribution in the inhibition by P, i.e. the possible formation of an off-pathway PE complex where P is not bound to the active site. The complete absence of (or very poor) binding of P to the free enzyme would indicate an uncompetitive inhibition where P only binds to the ES complex. This situation can be analysed by the global fitting strategy on the basis of Eq. (27) of Orsi and Tipton[4]. Similarly, the non-competitive (or mixed) inhibition situation can be analysed on the basis of Eq. (30) proposed in the same paper. But in the latter case, there are 2 inhibition constants (K_i and K'_i) and the number of experimental measurements should accordingly be increased.

Inhibition by the substrate

As in the other cases, we performed 18 simulations with K_m and $V = 1$ and $K_i = 0.33$, 1.0, 3.0 and 6.0 and added errors up to 2, 5 and 10%.

With $K_i = 0.33$ and 1.0, the v or $[P]/t$ *vs.* $[S]_0$ curves exhibited clear maxima while this was not the case with the higher K_i values.

Case 1: There is a clear maximum in the v or $[P]/t$ *vs.* $[S]_0$ curve.

Table S11 compares the results obtained with the help of Eq. (6) with real v values and 10, 30 or 60% substrate conversion and with maximum errors ranging from 2 to 10%.

Four major conclusions can be drawn from these results:

(1) There is no significant difference between the real initial rates (v) and the situations where up to 60% of the substrate is converted.
(2) With maximum errors of 2% the results are quite satisfactory although the SEs can be up to about 30%.
(3) With errors up to 5 and 10%, the results become much less satisfactory. The ranges of the determined parameter values become much larger and, with errors up to 10%, some absurd results can be obtained. In both cases, the SEs are sometimes larger than the values themselves. Note that the simulations performed with the real initial rate (v) values never yield better results than those done with large percentages of substrate conversion.
(4) An analysis based on the HW linearisation (without taking account of a possible inhibition by S) yields absurd results (negative K_m values).

In conclusion, if the experimental errors on the measurements of v or $[P]/t$ cannot be limited to 2%, a simple utilization of the integrated equation is unlikely to supply correct values of V, K_m and K_i. This is particularly true if K_i is smaller than K_m but even when $K_i = K_m$ the ranges are quite large (0.39–5.09 for K_m) when the errors are up to 10%. Even if the derived values themselves happen to be correct, the SEs are large as shown by the following example obtained with $K_i = 1.0$, 10% of substrate conversion and errors up to 5%: $V = 1.07 \pm 0.29$, $K_m = 1.03 \pm 0.40$ and $K_i = 0.90 \pm 0.34$!

In fact, it seems quite dangerous to try to derive the 3 parameters from only 7 measurements of v or $[P]/t$ as done here. Is it possible to obtain an approximate value for one of the parameters? What happens if the maximum of the v or $[P]/t$ *vs.* $[S]_0$ curve can help in supplying a fair approximation of K_i? In fact, derivatization of Eq. (8) shows that $([S]_0)_{max} = (K_m \cdot K_i)^{1/2}$. When $K_i > K_m$ the maximum might not be clearly visible within the range of S concentrations used in the simulations (see Table S13). When $K_i \leq K_m$, a maximum is clearly visible. Since a K_i value much lower than K_m would be exceptional (and have little physiological meaning), we used a lower limit of $K_i = 0.25$ km in our simulations.

In a first approach, we used fixed values of K_i (F) and replaced K_i by F in Eqs. (5) and (6) for the fitting to the simulated results. Table S13A shows the detailed results of the analysis of the simulations for (real) K_i values of 0.33 and 1.0. In all cases, the results are rather reasonable. For $K_i = 0.33$ (fixed $K_i = 0.7$), the V and K_m values are underestimated by factors of at most 2 for V and 3 for K_m. However, a situation where K_i is smaller than K_m is rather uncommon. For $K_i = 1.00$ (fixed $K_i = 1.5$), the underestimation is somewhat less marked (a factor 1.8 in the worst case). When the fixed K_i is smaller than the real one, both V and K_m are overestimated. We also performed the analysis with the help of the initial rate Eq. (5). The results (Table S13B) are in very good agreement with those obtained with the integrated Eq. (6). At 10% conversion, there is no significant difference between the results obtained with the two equations. At 30 and 60% conversion, the K_m values are somewhat higher with Eq. (5) as expected on the basis of Table S1. Note that in all cases, the ranges and the SEs are similar with both equations and the latter significantly much lower than when the 3 parameters are retrieved on the basis of the integrated equation.

In a second approach, we expressed K_i as a function of K_m ($K_i = n \cdot K_m$) and replaced K_i by $n \cdot K_m$ in Eq. (6). Results for 60% conversion of substrate and errors up to 10% are summarized in Table S14. The results are similar to those obtained in the first approach (Fixed K_i).

Finally, Table S15 compares the results of 6 fittings for individual values of the simulations according to the 3 strategies, Free K_i, Fixed K_i and $K_i = n \cdot K_m$ (in all cases, real $K_m = 1.0$, 60% substrate conversion and errors up to 10%). The simulations were chosen among those that gave the best or the worst results according to the "Free K_i" strategy. In all cases, the "Fixed K_i" and "$K_i = n \cdot K_m$" strategies yield excellent results and, with both approaches, the SEs are quite low when compared to those recorded with the "Free K_i" strategy that involves 3 unknown

parameters and where SEs can be larger than the calculated value. In conclusion, when $K_i \leq K_m$, choosing a K_i value on the basis of the maximum of the [P]/*t vs.* $[S]_0$ curve is a good approach.

Case 2: There is no clear maximum in the *v* or [P]/*t vs.* $[S]_0$ curve.

The simulations were performed for K_i values of 3.0 and 6.0. Here, an analysis based on the HW linearization (Eq. 1b, that does not take account of a possible inhibition by the substrate) does not yield absurd results (Table S16) but the SEs are very large and the plots generally exhibit an upward curvature that can however not be clearly visible when errors are up to 10% (see Fig. S1). Similarly, direct application of the integrated Eq. (1c) results in reasonable (if rather poor) approximations of *V* and K_m but again the SEs are large. These large SEs suggest that substrate inhibition occurs with a K_i/K_m ratio > 1. This can be easily verified by performing some measurement at larger $[S]_0$ concentrations. When fittings are performed with real *v* values according to Eq. (5) or with the integrated Eq. (6) (for 10, 30 or 60% of substrate conversion), the ranges of the *V*, K_m and K_i values are quite wide, some absurd results are recorded and, in all cases, the SEs are quite large, sometimes larger than the values themselves. With the "Fixed K_i" strategy, the results are significantly better. Note that the ranges of the K_m and *V* values are acceptable if one takes account of the systematic overevaluation of K_m when Eq. (5) is used (with $v = [P]/t$, see Table S1). Finally, similar results are obtained with the "$K_i = n \cdot K_m$" strategy (Table S14) that yields good *V*, K_m and K_i values with reasonable SEs. To confirm this, we compared the results of 6 fittings of individual values for $K_i = 3$ as above (Table S17) and the results show that the lowest SEs are always obtained with n = 2 or 4 but even if n = 6, the overevaluation of K_i never exceeds a factor 2.

In the experimental approaches, Tables 2 and 3 show the results of fitting the various equations to the raw data according to all the strategies described above (see below in the "Experimental Studies" section).

Experimental studies

All kinetics were recorded at 30 °C using a Specord 200 spectrophotometer (Analytik Jena, Germany) with the class C β-lactamase CMY-1. The enzyme was purified as described by Bauvois et al[5]. The experiments were conducted in 50 mM MOPS buffer (pH 7) containing 50 mM NaCl. The enzyme was diluted in the same buffer, supplemented with 50 µg/ml of BSA. Absorbance was monitored (2 pts/s) at 486 nm in a 2 mm quartz cuvette for nitrocefin ($\Delta\varepsilon 486nm = 15{,}000\ M^{-1}\ cm^{-1}$) and at 273 nm in a 1 cm quartz cuvette for cefalothin ($\Delta\varepsilon 273nm = -6300\ M^{-1}\ cm^{-1}$). The reaction time courses were monitored as previously described[1,5,6]. Briefly, the time points at which about 50 or 60% of the substrate was converted at the lowest concentration were first determined for cefalothin (74 s) and nitrocefin (152 s), respectively, and the absorbance values at these times were recorded for the other concentrations, ranging from 20 to 100 µM for cefalothin (enzyme concentration: 1.2 nM) and 50 to 600 µM for nitrocefin (enzyme concentration 0.36 nM).

Inhibition by P.

In a preliminary experiment, 300 µM cefalothin was completely hydrolysed by the enzyme before adding fresh cefalothin to a final concentration of 300 µM. About 30% inhibition was recorded when compared to a control sample under initial rate conditions. Similarly, with 100 µM hydrolysed cefalothin and 100 µM fresh substrate, the inhibition was about 17%.

The results are summarised in Table 2 (the raw data can be found in Table S18). The K_m and *V* values were computed as follows. Direct HMM and HW: with the [P]/t ratios after 74 s and Eqs. (1) (HMM) or (1b) (HW). Fixed K_p: with the [P] values after 74 s and the help of the integrated Eq. (3). Initial rates: with the [P] values after 10 s and the HMM equation. Note that the results obtained at the 2 lowest concentrations were not taken into account (absorbance variations too low).

The K_m and *V* values determined on the basis of the initial rates are in good agreement with those derived from the Fixed K_p strategy with the help of the integrated equation. Similarly, the (K_m)direct/(K_m)fixed ratio at Fixed $K_p = 50$ µM is 1.59, in good agreement with the expected value (1.56, Table S1 and Ref.[1]).

	K_m	SE K_m	SE/K_m (%)	*V* (µM/s)	SE *V*	SE/*V* (%)	R^2
Direct HMM	27.0	4.0	15	0.335	0.017	5.0	
Direct HW	28.4	4.0	14	0.341	0.015	4.5	
Fixed *Kp* 25 µM	16.0	2.5	16	0.332	0.016	4.8	0.960
30 µM	16.5	2.4	14.5	0.330	0.016	4.8	0.962
50 µM	17.4	2.4	13.7	0.327	0.014	4.3	0.967
70 µM	17.9	2.4	13.4	0.325	0.013	4.0	0.969
100 µM	18.2	2.3	12.6	0.324	0.013	4.0	0.971
150 µM	18.5	2.4	13.0	0.322	0.014	4.3	0.972
200 µM	18.6	2.3	12.4	0.322	0.012	3.7	0.973
Initial rate (HW)	16.2	1.8	11.1	0.360	0.008	2.2	
Initial rate (HMM)	16.1	1.8	11.2	0.360	0.014	4.0	

Table 2. Inhibition by the product, experimental data. For the "Direct" and Fixed K_p values, t = 74 s and 47.6% of the substrate were converted at the lowest $[S]_0$ value (20 µM). The initial rates were recorded over 10 s but the results at the 2 lowest concentrations (20 and 30 µM) were not taken into account (the absorbance variations were too low). The raw data can be found in table S18.

On the basis of these data, one can only say that $K_p \geq 50$ µM (the R^2 values no longer significantly increase above $K_p = 70$ µM) but K_p values in the 50–100 µM range are in better agreement with the results of the preliminary experiments although the errors are not significantly different with $K_p > 100$ µM. Figure 1 shows a fit of the [P]/t values vs $[S]_0$ after 74 s with the integrated equation and Fixed $K_p = 50$ µM yielding $K_m = 17.4$ µM and $V = 0.327$ µM/s ($k_{cat} = 270\ s^{-1}$) and $R^2 = 0.967$. Note that with Fixed K_p values lower than 25 µM, the errors on K_m become very large.

Inhibition by S.

A clear maximum in both the v_0 and [P]/t vs $[S]_0$ curves could be detected (see Fig. 2 and Table S19 for the raw data). As expected on the basis of the simulations (clear maximum), analysis according to the simple HW Eq. (1b) yielded absurd results: clear upward curvatures and negative K_m value for the [P]/*t* data (not shown). The results obtained with the other approaches and Eqs. (5) and (6) are summarised in Table 3. The K_m and V values were computed as follows: "Simple": direct application of Eq. (5) to the *v* or [P]/*t* values. Equation (5) was also utilised in the Fixed K_p and $K_i = n{\cdot}K_m$ approaches for the *v* values. For the [P]/*t* values after 152 s, the analysis rested on the integrated Eq. (6) in the Fixed K_i and $K_i = n{\cdot}K_m$ approaches.

With the *v* measurements, the highest R^2 values are obtained with the "simple", "Fixed K_i" ($K_i = 1100$–1300 µM) and "$K_i = n{\cdot}K_m$" (with n = 13) approaches that yield very coherent results: K_m: 73–84 µM, $V = 0.46$–0.49 µM/s

	v					**[P]/t (t = 152 s)**				
	K_m (µM)	V (µM/s)	K_i (µM)	R^2	$([S]_0)_{max}$ (µM)	K_m (µM)	V (µM/s)	K_i (µM)	R^2	$([S]_0)_{max}$ (µM)
Simple	73 ± 26	0.46 ± 0.07	1350 ± 770	0.949	347	66 ± 20	0.46 ± 0.06	880 ± 340	0.942	241
Integrated						32 ± 10	0.39 ± 0.04	1300 ± 600	0.917	204
Fixed *Ki* 500	146 ± 23	0.68 ± 0.04	500	0.883	270	66 ± 13	0.54 ± 0.03	500	0.734	182
700	112 ± 15	0.58 ± 0.025	700	0.921	280	50 ± 8.3	0.47 ± 0.017	700	0.844	187
900	94 ± 12	0.53 ± 0.02	900	0.939	291	42 ± 6.1	0.43 ± 0.014	900	0.893	194
1100	82 ± 10	0.49 ± 0.017	1100	0.947	310	36 ± 5.1	0.40 ± 0.012	1100	0.912	199
1300	74 ± 9.4	0.47 ± 0.016	1300	0.949	310	32 ± 4.6	0.39 ± 0.011	1300	0.917	205
$K_i = n{\cdot}K_m$										
n = 3	158 ± 14	0.71 ± 0.021	474 ± 42	0.874	274	108 ± 14	0.69 ± 0.032	324 ± 42	0.510	187
n = 5	126 ± 10	0.61 ± 0.015	630 ± 50	0.910	282	84 ± 9.7	0.60 ± 0.024	420 ± 49	0.645	188
n = 7	108 ± 8.5	0.57 ± 0.013	756 ± 60	0.928	286	72 ± 7.7	0.55 ± 0.019	504 ± 11	0.723	190
n = 10	93 ± 7.1	0.52 ± 0.011	930 ± 71	0.941	294	60 ± 6.0	0.50 ± 0.015	600 ± 60	0.798	190
n = 13	84 ± 6.4	0.49 ± 0.010	1090 ± 83	0.946	302	53 ± 5.0	0.48 ± 0.013	690 ± 65	0.843	191

Table 3. Inhibition by the substrate. Results obtained with the initial rates (*v* based on the absorbance variation after 10 s) are compared to those obtained with the [P]/*t* values after 152 s. « Simple » represents the direct application of Eq. (5) to the *v* or [P]/t measurements and $([S]_0)_{max}$ the calculated value for the maximum in the *v* or [P]/*t* vs $[S]_0$ curves. The "Fixed K_i" and "$K_i = n{\cdot}K_m$" strategies involve the utilisation of the integrated Eq. (6). The raw data can be found in Table S19.

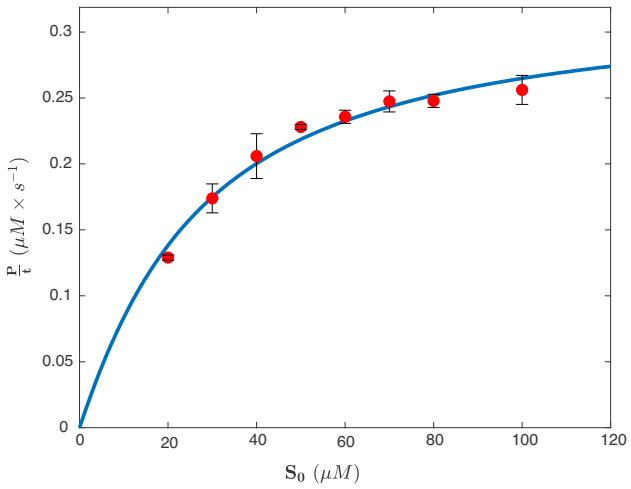

Fig. 1. Inhibition by P. Fit of the integrated Eq. (3) with Fixed $K_p = 50$ µM to the experimental data ([P]/*t* after 74 s, Table S18). When K_p is fixed, Eq. (3) contains only V and K_m as unknown parameters. According to this strategy, the retrieved values for K_m and V are 17.4 ± 2.4 µM and 0.33 ± 0.14 µM/s, respectively. $R^2 = 0.967$ (Table 2).

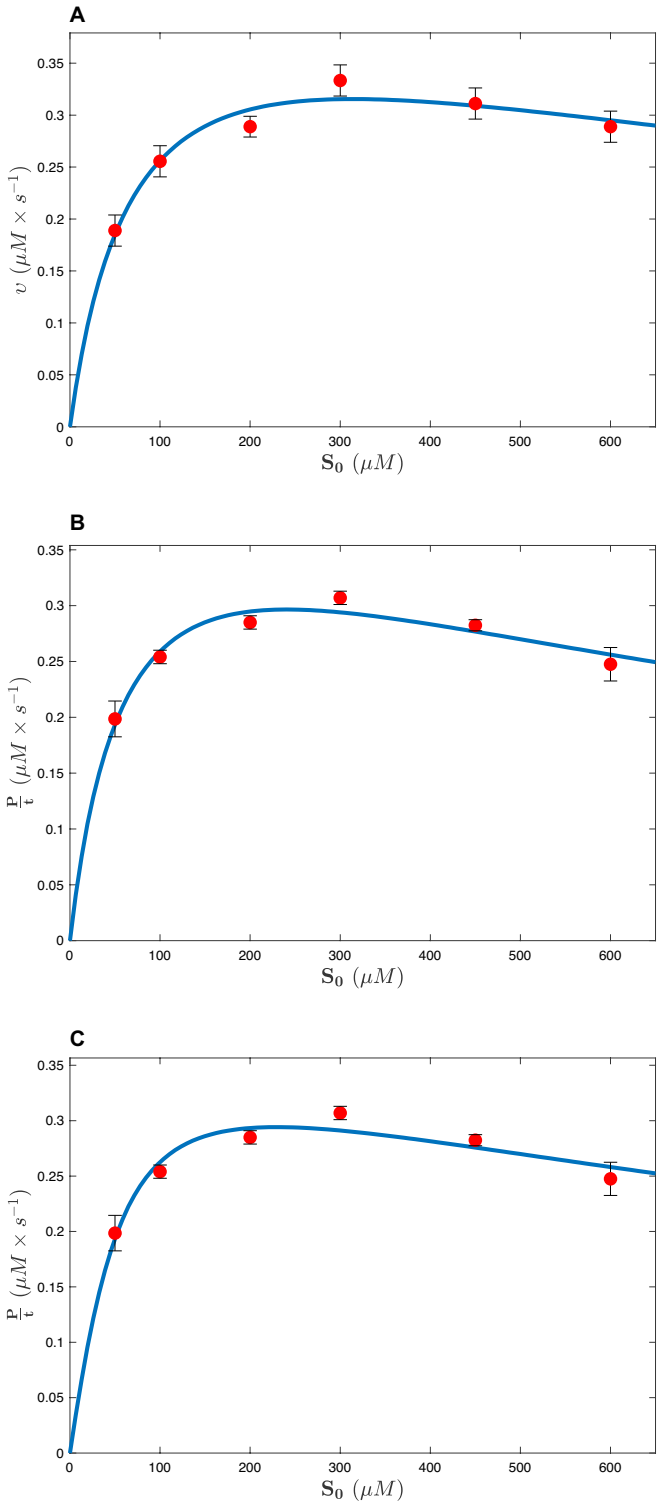

Fig. 2. Inhibition by S. (**A**) Initial rate data. The solid curve represents the fit of Eq. (5) to the experimental data, Table S19). The retrieved values for $K_{m,}$ V and K_i are 73 ± 26 μM, 0.46 ± 0.075 μM/s and 1350 ± 770, respectively. $R^2 = 0.949$ (Table 3, v simple). (**B**) [P]/t after 152 s. Simple [P]/t (Eq. 5 where [P]/t is wrongly assumed to represent v). The solid curve represents the fit of Eq. (5) to the experimental data (Table S19). The retrieved values for $K_{m,}$ V and K_i are 66 ± 20 μM, 0.46 ± 0.06 μM/s and 880 ± 340, respectively. $R^2 = 0.942$ (Table 3, [P]/t, simple). (**C**) [P]/t after 152 s. Integrated [P]/t (Eq. 6). The solid curve represents the fit of Eq. (6) to the experimental data (Table S19). The retrieved values for $K_{m,}$ V and K_i are 32 ± 10 μM, 0.39 ± 0.04 μM/s and 1300 ± 600, respectively. $R^2 = 0.917$ (Table 3, [P]/t, integrated).

and $K_i = 1100–1350$ µM. With [P]/*t* (60% conversion at $[S]_0 = 50$ µM) the highest R^2 values are obtained with the "simple", "integrated" and "Fixed K_i" ($K_i = 1100–1300$ µM) approaches. The "simple" results must be corrected according to Table 1 for 60% conversion, yielding $K_m = 36 \pm 11$ µM and $V = 0.39 \pm 0.05$ µM/s so that the 3 approaches yield again very coherent results: $K_m = 32–36$ µM and $V = 0.39$ µM/s. The *V* values are in excellent agreement with those determined according to the *v* measurements while the K_m values are somewhat lower with [P]/*t*. This might just be due to the experimental errors. Indeed, the "simple" approach yields $K_m = 73 \pm 26$ µM for *v* and 36 ± 11 µM for [P]/*t* (after correction) which can be considered as a fair agreement. *V* values are respectively 0.46 (v_0), 0.39 (simple [P]/*t*, after correction) and 0.39 µM/s (integrated [P]/t) yielding $k_{cat} = 1100$ to 1300 s^{-1}. The "simple" approach also yields the best $([S]_0)_{max}$ values. Note that the $([S]_0)_{max}$ value is somewhat higher with the true initial rates. For K_i, one can safely assume a value in the 900 to 1300 µM range.

Figure 2 shows the fittings of the "simple" (*v* and [P]/*t*) and integrated equations ([P]/*t*) to the experimental data.

Finally, note that the observation of a clear maximum in the *v* and [P]/*t* vs $[S]_0$ curves was not predicted by the simulations but this is due to the fact that in the simulations, the highest $[S]_0$ value was only 2.45 K_m while it was ≥ 10 K_m in the experiments.

Discussion

Inhibition by the product was analysed according to various approaches. Note that our analysis rested on the assumption of a competitive inhibition (see below).

The simulations show that the most rigorous approach is probably the "Global fitting" strategy with at least 15 data points obtained with various product concentrations added at time zero (in our simulations, the $[S]_0$ values were centred around that of K_m but in less favourable cases, the number of measurements should probably be increased). On the other hand, if one wants to reduce the number of measurements to a minimum, an independent determination of K_p presents a major improvement. Even when this independently measured K_p value is not very accurate, the obtained *V* and K_m values are excellent. In summary (with $[P]_0 = 0$), when the K_p/K_m ratio is ≥ 1 (but even when this ratio is 0.25), *V* and K_m can be determined in a very satisfactory way but not the K_p value. All these measurements can involve the conversion of a large proportion of S.

Our experimental data confirm these theoretical predictions. With up to 50% of substrate converted at the lowest $[S]_0$ concentration and 1 single time point for each of the $[S]_0$ concentrations, excellent values of *V* and K_m were retrieved and the agreement between the various strategies was striking. A determination of the same parameters on the basis of initial rate measurements yielded the same results.

There is however an important caveat. As mentioned above and confirmed by the experimental data, the K_p value cannot be evaluated in a satisfactory way. Similarly, the type of inhibition (competitive, uncompetitive or non competitive) cannot be determined. This would require to perform experiments with various $[P]_0$ concentrations and to analyse (global fitting) the results according to the three models. In a further contribution, we plan to analyse the possibility of performing these studies with a large proportion of substrate converted.

Yun and Suelter[7] proposed a method to determine the 3 parameters on the basis of the integrated equation but their approach rests on the analysis of complete time-courses. Moreover, they only considered very small errors on $[S]_0$ (0.2 and 0.5%) and on the readings in the progress curves (0.2%). As shown here, when larger errors are introduced, utilisation of the integrated equation with 3 parameters can become quite problematic.

Inhibition by the substrate was examined by Clark and Jowett[8] who proposed a method to evaluate the initial rates in this situation. Again, their analysis rests on the determination of time-courses and they do not take account of the possible experimental errors.

Our proposed strategy was to introduce a K_i value based on the position of the maximum in the [P]/t *vs.* $[S]_0$ curve. In this strategy, K_i can be "fixed" or replaced by $n \cdot K_m$. This decreases the number of parameters to be determined from 3 to 2. The best value for Fixed K_i or n is then the one that results in the lowest R^2. In our experiments, when 60% of the substrate was converted at the lowest $[S]_0$, the "simple [P]/t" and "integrated [P]/t" strategies yielded the best and coherent results (highest R^2) also in agreement with Fixed $K_i = 1300$ µM. As expected, the SEs were lower with Fixed K_i and $K_i = n \cdot K_m$ but, in the latter case, the calculated $[S_0]_{max}$ was somewhat too low. We want to stress that the value of $[S_0]_{max}$ is a very important experimental result to take account of.

In conclusion, reliable estimations of *V* and K_m can be obtained when a large proportion of the substrate is converted both when inhibition by P or S occurs. However, in the former case, additional experiments are certainly needed to determine the type of inhibition and the exact K_p value. In the latter, the K_i value can remain somewhat less reliable.

Finally, we want to stress the fact that it is probably easier to obtain accurate [P]/*t* than *v* values. Indeed, evaluation of the latter often involves the utilisation of regression methods[9] that can introduce additional errors or the conversion of a very small proportion of the substrate which can result in increased inaccuracies if these measurements are performed not well above the limit of the detection method.

Data availability

The complete data can be found in https://doi.org/https://doi.org/10.5281/zenodo.7528423.

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Author contributions

JMF designed the study and wrote the paper. OV performed and analysed the simulations, RM performed the experiments. WV and AM designed the study and wrote the paper (together with JMF). All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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