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Mechanistic Kinetic Model for Symmetric Carboligations Using Benzaldehyde Lyase

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ABSTRACT: For reactions using thiamine diphosphate (ThDP)-dependent enzymes many empirically-derived kinetic models exist. However, there is a lack of mechanistic kinetic models. This is especially true for the synthesis of symmetric 2-hydroxy ketones from two identical aldehydes, with one substrate acting as the donor and the other as the acceptor. In this contribution, a systematic approach for deriving such a kinetic model for thiamine diphosphate (ThDP)-dependent enzymes is presented. The derived mechanistic kinetic model takes this donor–acceptor principle into account by containing two K_m -values even for identical substrate molecules. As example the stereoselective carbon–carbon coupling of two 3,5-dimethoxy-benzaldehyde molecules to (*R*)-3,3',5,5'-tetramethoxy-benzoin using benzaldehyde lyase (EC 4.1.2.38) from *Pseudomonas fluorescens* is studied. The model is derived using a model-based experimental analysis method which includes parameter estimation, model analysis, optimal experimental design, in silico experiments, sensitivity analysis and model revision. It is shown that this approach leads to a robust kinetic model with accurate parameter estimates and an excellent prediction capability.

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KEYWORDS: thiamine diphosphate; benzaldehyde lyase; kinetic modeling; parameter estimation; optimal experimental design; in silico experiments

Introduction

Thiamin diphosphate (ThDP)-dependent enzymes, such as benzaldehyde lyase (BAL, EC 4.1.2.38), benzoylformate decarboxylase (BFD, EC 4.1.1.7) and pyruvate decarboxylase (PDC, EC 4.1.1.1), catalyze carboligase reactions with excellent enantioselectivity (Demir et al., 2001b; Iding et al., 2000; Pohl et al., 2002). This formation of carbon–carbon bonds constitutes one of the key transformations in synthetic organic chemistry (Faber and Kroutil, 2005). Especially the production of chiral hydroxy ketones is of commercial interest since they are important building blocks of drugs and natural products (Demir et al., 2001a).

A reaction mechanism for the decarboxylation has been proposed for PDC (Kluger, 1987) and BFD (Reynolds et al., 1988; Iding et al., 2000; Weiss et al., 1988). According to these investigations, the reaction is catalyzed directly at the cofactor ThDP with an enamine-carbanion as intermediate. A similar mechanism with corresponding ThDP intermediates was later proposed for the carboligation using BAL (Demir et al., 2001b). It is assumed that the enzymes act only as stabilizers of the zwitter-ionic state of the intermediate (Jordan, 2003). Using isoelectric analogues of ThDP, it has been confirmed that the cofactor plays the crucial role in the reaction mechanism (Leeper et al., 2005). Moreover, X-ray diffraction analysis has revealed that among these enzymes, their cofactor orientation is remarkably similar (Mosbacher et al., 2005). To optimize processes using ThDP-dependent enzymes, it is essential to derive a kinetic model that describes the kinetics of these enzymes in a mechanistically correct manner. According to Vasic-Racki et al. (2003) such a model contributes to an increase in knowledge about the process, which helps to identify optimal operating conditions. Most ThDP-dependent enzymes follow a similar mechanism. Among this group of enzymes, BAL is of special

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interest. BAL is a very active catalyst that is able to form and also to cleave chiral hydroxy ketones. Other ThDP-dependent enzymes, such as BFD, are not able to perform this cleavage due to steric hindrance (Knoll et al., 2006). Therefore, in order to fit the derived kinetic model, BAL is chosen.

BAL was discovered by Gonzalez and Vicuna (1989), who had previously observed that *Pseudomonas fluorescens* is able to grow on media with benzoin or anisoin as the only carbon source (Gonzalez et al., 1988). BAL is a homo-tetramer of four identical subunits of 563 amino acid residues, corresponding to a molecular mass of 58,919 Da. The cofactors ThDP and Mg^{2+} are bound at the interface of a dimer, such that one binding site is formed by two monomers. Nevertheless, the tetrameric enzyme contains four active centers (Mosbacher et al., 2005). BAL has a broad substrate spectrum of differently substituted benzaldehydes. According to Dünkemann et al. (2002) one substrate molecule acts as a donor whereas another acts as an acceptor.

To date, this donor–acceptor principle has been applied only for the synthesis with two different substrates, namely *A* and *B*. By varying the concentration of *A*, while *B* is in surplus, and vice versa, the Michaelis constants, K_{mA} and K_{mB} , respectively, can be determined by initial rate measurements. However, the special situation of the synthesis with two identical substrate molecules *A* to a symmetric benzoin renders this approach infeasible. Therefore, the occurrence of different K_m -values, for both the donor and the acceptor, has been ignored in previous studies that assume that only a single K_m -value exists (e.g., Hildebrand et al., 2007; Stillger et al., 2006). Motivated by this lack of argument, a novel mechanistic kinetic model with two independent K_m -values is derived for the first time in this contribution.

To fit this mechanistic kinetic model, progress curve analysis is performed, where concentrations are monitored for longer time periods, at most until the thermodynamic equilibrium is reached. Progress curve analysis should be preferred since it can provide more information than initial rate analysis (Duggleby, 2001). For example, effects occurring after a longer period of time, such as enzyme deactivation, can only be detected this way. Moreover, this approach is of industrial interest since for technical applications enzymatic reactions are used within much longer time periods until high conversions are reached. Progress curve analysis requires special software for dynamic simulation and parameter estimation. Although this software is readily available nowadays, this approach is hardly being used in the biocatalytic community. One reason might be that it is still difficult to estimate parameters of non-linear models, if the quality of the initial guess is poor. To overcome this difficulty, Vasic-Racki et al. (2003) proposed to conduct first initial rate measurements to get reasonable initial guesses for the kinetic parameters. However, initial rate measurements are laborious and not feasible in the case of identical substrates. Another approach that can help

getting good initial parameter guesses has been presented by Brendel et al. (2006). In that study a so-called incremental identification method is presented that efficiently computes initial parameter guesses based on the model and the available measurement data. The method has recently been applied to a biocatalytic reaction system, namely the formate oxidation using formate dehydrogenase by Michalik et al. (2007).

In this work, the new mechanistic kinetic model for the condensation reaction of two identical substrates with BAL was fitted directly to progress curves without preceding initial rate measurements. The predictability of the derived model was analyzed using advanced statistics tools. Optimal experiments were designed to estimate the parameters precisely. Moreover, the effects of reasonable assumptions on the model predictability have been checked.

Materials and Methods

Experimental

All chemical substances were of analytical grade and purchased by Sigma–Aldrich (Deisenhofen, Germany). 3,5-Dimethoxy-benzaldehyde (DMBA) was used as substrate yielding the product (*R*)-3,3',5,5'-tetramethoxybenzoin (TMB). BAL was fermented in *E. coli* as a fusion protein with His-tag and purified with affinity chromatography. The enzyme was stored as a lyophilisate. The applied assay contained 50 mM KPi buffer and both cofactors, 0.25 mM $MgCl_2$ and 0.25 mM ThDP. To increase the solubility of the aromatic compounds, 30% (v/v) of the cosolvent dimethylsulfoxide (DMSO) was added, which also has a stabilizing effect on the enzyme (Dominguez de Maria et al., 2006). Finally, the pH value was adjusted to 8.5 and the ionic strength to 150 mM. The pH value is one unit lower than the activity optimum of 9.5 observed by Dominguez de Maria et al. (2006), but leads to a higher stability of the enzyme. Consequently, the pH value of 8.5 was chosen as a compromise between stability and activity.

Since BAL (*Pseudomonas fluorescens* Biovar I) is a tetramer of four identical subunits, the activity is related to one subunit. Assuming pure enzyme without additional proteins, the molar concentration of the subunit is calculated by dividing the weighted lyophilisate by the molecular weight (59,800 Da) of the subunit including the His-tag (Janzen et al., 2006).

The reaction kinetic measurements were conducted at 25°C using a fluorimeter (PerkinElmer, LS55, [Waltham](#)^{Q3}). The concentration of the substrate DMBA was monitored by exciting at 360 nm and recording the fluorescence intensity at 470 nm. Nine progress curves were measured (Table I, experiments A-I). The initial substrate concentration was varied between 1.5 and 3 mM. Owing to the limited solubility of the product, the initial substrate concentration could not exceed 3 mM. Otherwise, the concentration of the formed product would have exceeded 1 mM, where the

Table 1. Initial experimental conditions (A–I), optimal design for in silico experiments (J–Q) and optimal design for in silico experiments using the simplified kinetic model (R–Y).

Experiment	A_0 (mM)	P_0 (mM)	E (mM)	Duration (s)
A	3.00	0	8.33E–05	1,500
B	2.75	0	8.33E–05	1,500
C	2.50	0	8.33E–05	1,500
D	2.25	0	8.33E–05	1,500
E	2.00	0	8.33E–05	700
F	1.50	0	8.33E–05	1,000
G	3.00	0	4.17E–05	3,000
H	2.75	0	4.17E–05	3,000
I	2.50	0	4.17E–05	3,000
J	0.585	0.000	1.25E–04	360
K	0.000	2.300	1.25E–04	640
L	0.585	0.000	1.25E–04	360
M	0.900	0.050	1.25E–04	360
N	4.200	0.000	1.25E–04	340
O	0.000	3.600	1.25E–04	292
P	2.200	1.400	1.25E–04	325
Q	0.050	0.100	1.25E–04	50
R	0.380	0.000	1.25E–04	380
S	0.000	4.800	1.25E–04	620
T	0.410	0.000	1.25E–04	410
U	2.840	0.310	1.25E–04	380
V	3.000	0.000	1.25E–04	320
W	0.000	4.300	1.25E–04	292
X	2.370	0.160	1.25E–04	310
Y	0.000	0.210	1.25E–04	50

For the optimal experimental design the degrees of freedom were the initial substrate concentration A_0 (limits: 0–5 mM), the initial product concentration P_0 (limits: 0–5 mM) and the measurement duration t (limits: 50–640 s).

product starts to precipitate. The enzyme concentrations have been chosen sufficiently high to prevent enzyme deactivation before equilibrium was reached. This effect has been investigated earlier (data not shown). As long as no significant enzyme deactivation occurs, the enzyme concentration can be considered to be constant in each single dynamic simulation (Selwyn, 1965). Therefore, two different enzyme concentrations are sufficient for the task of model identification. The measurements have been performed until equilibrium—indicated by a constant fluorescence signal—was reached.

Model Identification Approach

The procedure applied for this study is illustrated in Figure 1. It follows the model-based experimental analysis (MEXA) approach described by Marquardt (2005). Based on initial knowledge about the enzyme mechanism, a kinetic model is developed (1), which takes all micro-reaction steps into account. This model is fitted to experimental data (2) and the quality of the estimated parameters is evaluated statistically in terms of the confidence regions of the parameters and the correlation matrix (Bard, 1974) (3). Moreover, confidence ellipsoids can be analyzed, which visualize the correlation between a pair of parameters

(Franceschini^{Q4} et al., 2007) and residuals can be plotted, which help to detect anomalies not predicted by the kinetic model (Bard, 1974; Cornish-Bowden, 2001). To detect the sensitivities of the model prediction, parametric sensitivity analyses can be performed.

If a kinetic model is not able to predict the experimental data satisfactorily, the model has to be revised in order to include more phenomena (Marquardt, 2005) (4). However, in many cases, the model can predict the experimental data quite well, but the parameters cannot be estimated with sufficient precision. In general, there are two possible reasons for this. First, the experimental data can be insufficient for estimating the parameters precisely. This is especially the case if the experimentally accessible region is rather limited, as, for example, due to low solubilities of the reactants. In this case, the model can, in principle, be identified by expanding the experimental limitations. Model-based optimal experimental design techniques (5) and in silico experiments (6) can be used to identify the necessary experimental region (Walter and Pronzato, 1990). The second reason for unsatisfactory parameter estimates is a model being too complex. The model is then over-specified. In this case, the model parameters are never identifiable, even after conducting optimal in silico experiments. In this case, the model has to be revised in order to reduce the model complexity (4).

For the purposes of modeling and dynamic simulations the software package gPROMS (version 3.0.2, Process System Enterprise Ltd., London, UK) was used. The addition of white noise for the in silico experiments and the sensitivity analysis were carried out using Matlab (version 7.3, The MathWorks Inc., Natick^{Q5}).

Results and Discussion

The model reaction for the derivation and experimental verification of the kinetic model for BAL is the carbonylation of 3,5-dimethoxy-benzaldehyde (DMBA) to (*R*)-3,3',5,5'-tetramethoxy-benzoin (TMB) (Fig. 2A). Initial experiments have shown that the substrate DMBA, which is substituted in meta-position, can act both as acceptor and donor forming the symmetric TMB.

Derivation of the Mechanistic Kinetic Model

The mechanism proposed by Demir et al. (2001b) was adapted for the synthesis of TMB. In Figure 2B, the three main micro-reaction steps for constructing the kinetic model are illustrated. The summarization and proton transfer steps are assumed to be very fast and thus not rate limiting. The micro-reaction constants k_i denote the rate constants for the micro-reaction steps. All these micro-reactions are reversible. Since two substrate molecules form one product molecule, the investigated reaction is denoted to follow an ordered bi-uni reaction mechanism (Cleland,

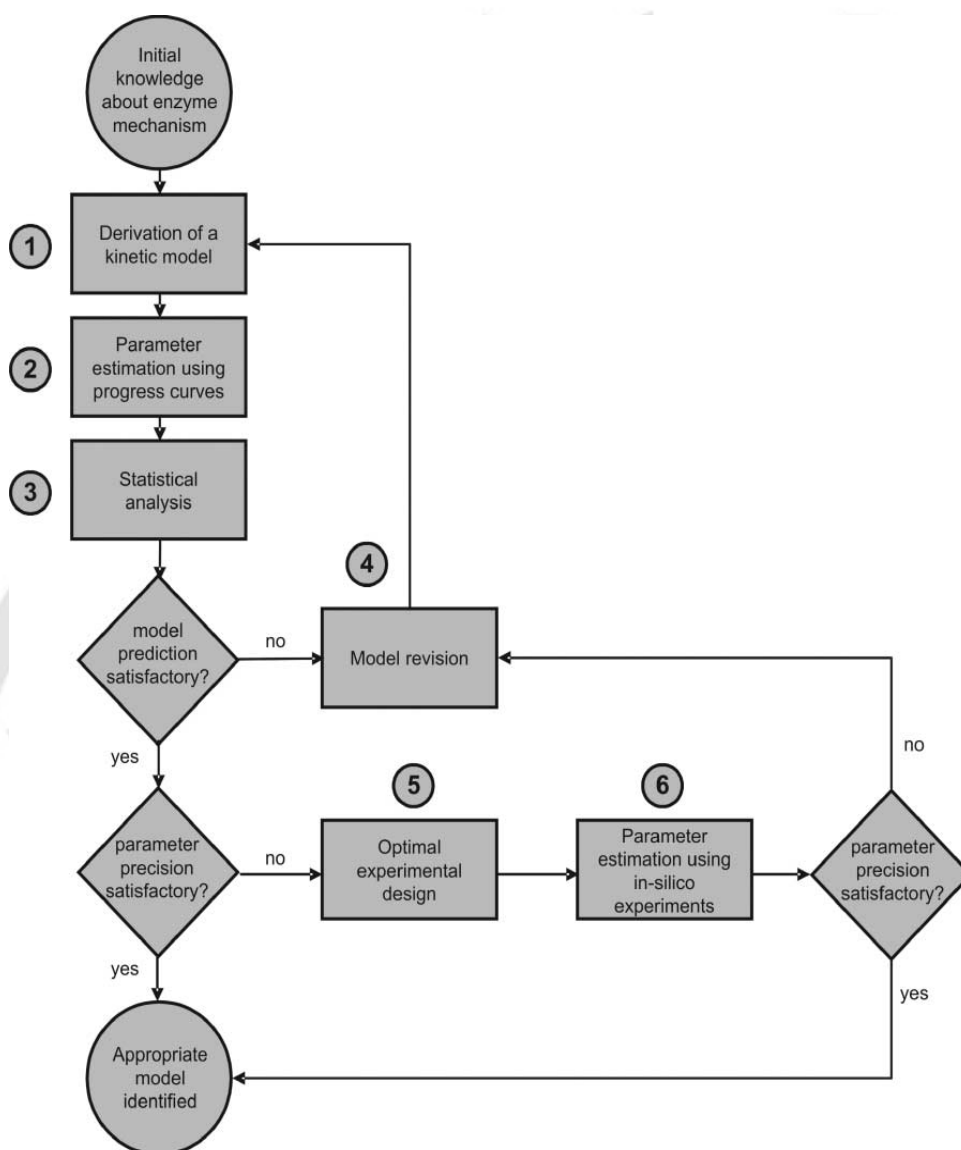


Figure 1. Applied procedure for deriving the kinetic model.

1963). This can be written in Cleland’s notation as depicted in Figure 2C. To distinguish between the first and the second binding substrate, they are denoted with *A* and *B*, respectively. *E* denotes the enzyme with the bound cofactors ThDP and Mg²⁺. As these cofactors are bound to the enzyme, the complex of free enzyme and cofactors can be treated as one single species. In the first reaction, the substrate *A* binds to the enzyme *E* forming the enzyme–substrate complex *EA*. After this, *B* binds to *EA* forming the ternary *EAB* complex. The enzyme is then recovered by forming the product *P*.

Applying the method of King and Altman (1956) Equation (1) was derived. This rate equation is mechanistically correct since it takes all micro-reaction steps into account including the inevitable competitive inhibitions of the forward reaction by *P* and of the reverse reaction by *A*. Moreover, the reverse reaction is considered. Consequently,

this rate law is valid for ordered bi–uni reactions under all concentrations of the reactants. Therefore, it should be preferred over multiplying simple Michaelis–Menten equations for all substances as it was done in previous publications (e.g., Hildebrand et al., 2007; Stillger et al., 2006)

$$v = -\frac{dA}{dt} = -\frac{dB}{dt} = \frac{dP}{dt}$$

$$= \frac{\frac{k_{catf}}{k_{iA}k_{mB}} \left(AB - \frac{P}{K_{eq}} \right)}{1 + \frac{A}{K_{iA}} + \frac{K_{mAB}}{K_{iA}K_{mB}} + \frac{AB}{K_{iA}K_{mB}} + \frac{P}{K_{mP}} + \frac{BP}{K_{mP}K_{iB}}} E \quad (1)$$

Equation (1) shows that the constructed model contains seven parameters, whereas only six micro-reaction constants exist. This means that one parameter is dependent on the

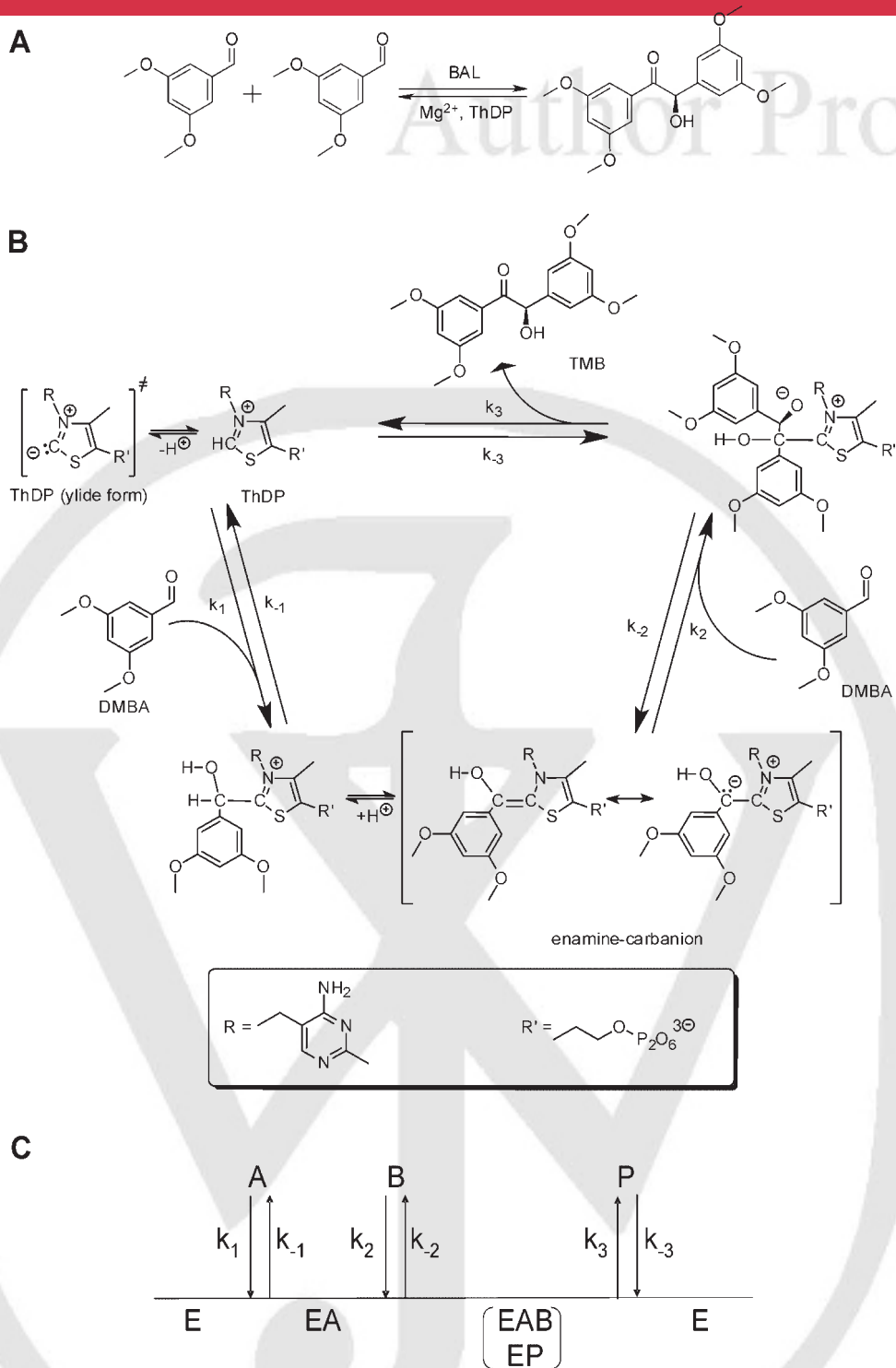


Figure 2. Synthesis reaction of 3,5-dimethoxy-benzaldehyde (DMBA) to (*R*)-3,3',5,5'-tetramethoxy-benzoin (TMB) (A). Reaction mechanism of BAL for the TMB synthesis adapted from Demir et al. (2001b) (B). Reaction mechanism of a bi-uni-reaction according to Cleland (1963) (C).

others. To detect this redundancy, the method of Straathof and Heijnen (1996) was applied and thus Equation (2) was identified. Table II lists the definitions of the remaining independent parameters

$$K_{iB} = \frac{K_{mB}K_{iA}}{K_{mA} \left[1 - \left(\frac{K_{mA}}{K_{iA}} - 1 \right) \frac{K_{mP}}{K_{eq}K_{mB}K_{iA}} \right]} \quad (2)$$

Besides K_{iB} there are two more dependent model parameters, k_{catr} and K_{iP} , which are functions of the independent parameters (Eqs. 22 and 23 in Appendix A). The thermodynamic equilibrium constant is defined as follows:

$$K_{eq} = \frac{P}{A^2} \quad (3)$$

Table II. Definitions of the model parameters for the derived mechanistic kinetic model and the simplified kinetic model.

Parameter	Mechanistic model	Simplified model	Meaning	Unit
k_{catf}	k_3	k_3	Maximum turnover number	s^{-1}
K_{eq}	$\frac{k_1 k_2 k_3}{k_{-1} k_{-2} k_{-3}}$	$\frac{k_1^2 k_3}{k_{-1}^2 k_{-3}}$	Equilibrium constant	mM^{-1}
K_{mA}	$\frac{k_3}{k_1}$	$\frac{k_3}{k_1}$	Affinity constant of A to E	mM
K_{mB}	$\frac{k_{-2} + k_3}{k_2}$	$\frac{k_{-1} + k_3}{k_1}$	Affinity constant of B to EA	mM
K_{mP}	$\frac{k_{-1}(k_{-2} + k_3)}{k_{-3}(k_{-1} + k_{-2})}$	—	Affinity constant of P to E	mM
K_{iA}	$\frac{k_{-1}}{k_1}$	—	Dissociation constant of EA	mM

If, in contrast, the reaction is modeled as uni–uni reaction, the thermodynamic equilibrium constant becomes:

$$K_{\text{eq}} = \frac{P}{A} \quad (4)$$

As a result, uni–uni models predict a wrong thermodynamic equilibrium, if different substrate concentrations are used. Thus, they can only be used for modeling initial rates and should not be used for processes, in which high conversions are intended.

With the definitions of the model parameters, the micro-reaction constants can be calculated (Appendix A). In the investigated special case of two identical substrates, the concentration of A and B are always equal ($A = B$). So Equation (1) is transformed to Equation (5):

$$v = -\frac{1}{2} \frac{dA}{dt} = \frac{dP}{dt} = \frac{\frac{k_{\text{catf}}}{K_{\text{iA}} K_{\text{mB}}} \left(A^2 - \frac{P}{K_{\text{eq}}} \right)}{1 + \frac{A}{K_{\text{iA}}} \left(1 + \frac{K_{\text{mA}}}{K_{\text{mB}}} \right) + \frac{A^2}{K_{\text{iA}} K_{\text{mB}}} + \frac{P}{K_{\text{mP}}} + \frac{AP}{K_{\text{mP}} K_{\text{iB}}}} E \quad (5)$$

Parameter Estimation

Nine experiments with varying experimental conditions according to Table I (experiments A–I) have been

conducted. The experimental data of these experiments have been used to estimate the parameters of the model given in Equation (5). After the parameter estimation step, the model is able to reproduce the experimental data accurately (data shown in the Supplementary Online Material). However, the precision of the parameter estimates is quite diverse (Table III). The thermodynamic equilibrium constant K_{eq} can be estimated very precisely. The accuracy of the maximum turnover number k_{catf} is also acceptable. All other kinetic parameters have unacceptably high inaccuracies since the confidence intervals are much larger than the estimated values. Hence, these parameters cannot be considered to be identifiable under the present experimental conditions. This hypothesis was strengthened by the obtained correlation matrix (Table IVA). Most kinetic parameters are highly correlated and, therefore, cannot be estimated independently. To illustrate the correlations between two parameters, confidence ellipsoids were drawn (Fig. 3). The semi-axes of the ellipsoid for the parameters K_{eq} and k_{catf} are almost parallel to the coordinate axes, which indicates a low correlation of both parameters. In contrast to this, the confidence ellipsoid of K_{mP} and K_{iA} implies a strong positive correlation of these two parameters. Thus, they cannot be estimated independently from each other. Moreover, the region of the 95% confidence ellipsoid of K_{mP} and K_{iA} also includes negative values for the kinetic parameters. This highlights the unreliability of these estimates since only positive kinetic parameters are possible.

According to the procedure described in Model Identification Approach Section, two possible reasons for unidentifiable parameters exist. The experimental data might contain

Table III. Parameter estimates and 95% confidence intervals using the derived mechanistic kinetic model (3rd column), after performing optimal in silico experiments (4th column), using the simplified kinetic model (5th column), and after performing optimal in silico experiments using the simplified kinetic model (6th column).

Parameter	Unit	Mechanistic model		Simplified model	
		Experiments A–I	Experiments J–Q	Experiments A–I	Experiments R–Y
k_{catf}	s^{-1}	46.123 ± 1.981	46.153 ± 0.047	45.443 ± 0.350	45.438 ± 0.038
K_{eq}	mM^{-1}	3.934 ± 0.034	3.936 ± 0.004	3.965 ± 0.028	3.989 ± 0.005
K_{mA}	mM	0.050 ± 0.852	0.050 ± 0.005	0.012 ± 0.006	0.010 ± 0.0002
K_{mB}	mM	0.005 ± 1.006	0.007 ± 0.007	0.047 ± 0.014	0.042 ± 0.0005
K_{mP}	mM	0.975 ± 56.645	1.205 ± 0.605	—	—
K_{iA}	mM	0.990 ± 242.333	0.647 ± 0.803	—	—

Table IV. Correlation matrix of the estimated parameters using the derived mechanistic kinetic model (A), after performing optimal in silico experiments (B), using the simplified kinetic model (C), and after performing optimal in silico experiments using the simplified kinetic model (D).

	k_{catf}	K_{eq}	K_{iA}	K_{mA}	K_{mB}	K_{mP}
A						
k_{catf}	1.00	-0.13	-0.86	-0.83	0.86	-0.64
K_{eq}		1.00	-0.10	-0.12	0.10	-0.34
K_{iA}			1.00	1.00	-1.00	0.93
K_{mA}				1.00	-1.00	0.94
K_{mB}					1.00	-0.93
K_{mP}						1.00
B						
k_{catf}	1.00	0.15	-0.63	-0.40	0.64	-0.03
K_{eq}		1.00	-0.05	0.05	0.06	0.50
K_{iA}			1.00	0.94	-1.00	0.56
K_{mA}				1.00	-0.93	0.64
K_{mB}					1.00	-0.55
K_{mP}						1.00
C						
k_{catf}	1.000					
K_{eq}		1.000	0.535	0.691		0.676
K_{mA}			1.000	0.403		0.386
K_{mB}				1.000		1.000
D						
k_{catf}	1.000					
K_{eq}		1.000	-0.620	0.079		0.036
K_{mA}			1.000	-0.527		-0.513
K_{mB}				1.000		0.999
K_{mP}						1.000

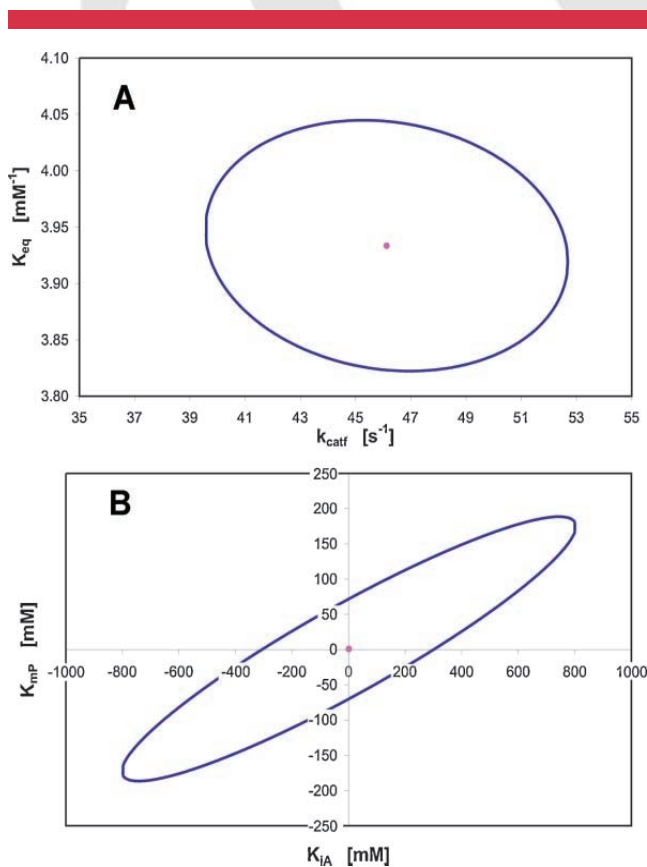


Figure 3. Ninety-five percent confidence ellipsoids for $K_{\text{eq}}/k_{\text{catf}}$ (top) and K_{mP}/K_{iA} (bottom).

insufficient information or the model structure might be too complex. To check whether this poor parameter precision is due to the tight experimental limitations, eight optimal experiments were designed. The degrees of freedom for the optimal experimental design were the initial concentrations of the substrate and the product and the experiment duration. The E-optimality criterion was used as the objective function (Walter and Pronzato, 1990). Reasonable limits have been used for all degrees of freedom. The aim was to determine whether the model parameters are generally not identifiable or whether they are identifiable under different experimental conditions, such as different substrate concentrations. Moreover, the addition of product at the beginning of the reaction was investigated, which was expected to cause a higher accuracy of the parameter estimates.

The designed optimal experiments are listed in Table I (experiments J–Q). These experiments confirm the assumption that it is advantageous to perform also measurements with initial product concentrations. Interestingly, the designed experiments contain quite different time scales. An explanation for this could be that the longer lasting experiments are optimizing the estimation accuracy of the equilibrium constant K_{eq} , whereas the shorter ones focus on the kinetic parameters in the beginning of the experiment such as k_{catf} .

Since the experimental limits could not be expanded sufficiently, the optimal experiments were carried out in silico. Therefore, simulations with the fitted mechanistic

kinetic model (Eqs. 2 and 5) and the conditions as determined by the optimal experiments were carried out. The simulated data for the substrate concentration were disturbed with white noise with a constant standard deviation of 0.001 mM. This low noise level, compared to the experimental error of about 0.05 mM, was used to assure that the experimental error was not the reason for a potential unidentifiability of the kinetic parameters. The obtained data were then treated as “real” experimental data for fitting the model. These data can be considered as taken under ideal conditions, which means that no solubility problems exist, enantiopure product is available and the measurement technique is highly precise.

The obtained parameter estimates show much smaller confidence regions which indicates that the experimentally accessible region has indeed been too limited in the first set of experiments and that the measurement technique is not sufficiently precise (Table III). The confidence ellipsoid for K_{mP} and K_{iA} (Fig. 4) illustrates that the confidence region decreased remarkably compared to the previous confidence ellipsoid of these parameters depicted in Figure 3B. On the other hand, the confidence region for K_{iA} still reaches negative values. Moreover, the correlation of K_{mP} and K_{iA} is still very high. High correlations between the kinetic parameters are also observed for other parameters (Table IVB). Thus, even if the in silico experiments described above are used and the model is assumed to be correct some model parameters can still not be estimated with sufficient accuracy. Therefore, it can be concluded that the tight experimental conditions are not the main reason for the low parameter precision, but a too complex model with too many parameters.

Before revising the model, it is reasonable to investigate why these parameters cannot be identified. Thus, in order to detect how sensitive the parameters of the derived kinetic model are, a dynamic sensitivity analysis was performed. Figure 5A depicts the normalized sensitivities of the model parameters, which were obtained by using Equation (6)

$$S_i = \frac{\theta}{A} \frac{\partial A}{\partial \theta} \quad (6)$$

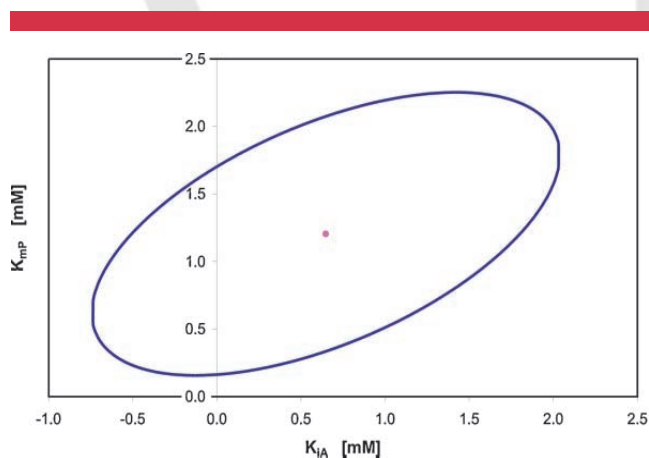


Figure 4. Ninety-five percent confidence ellipsoid for K_{mP}/K_{iA} after performing in silico experiments.

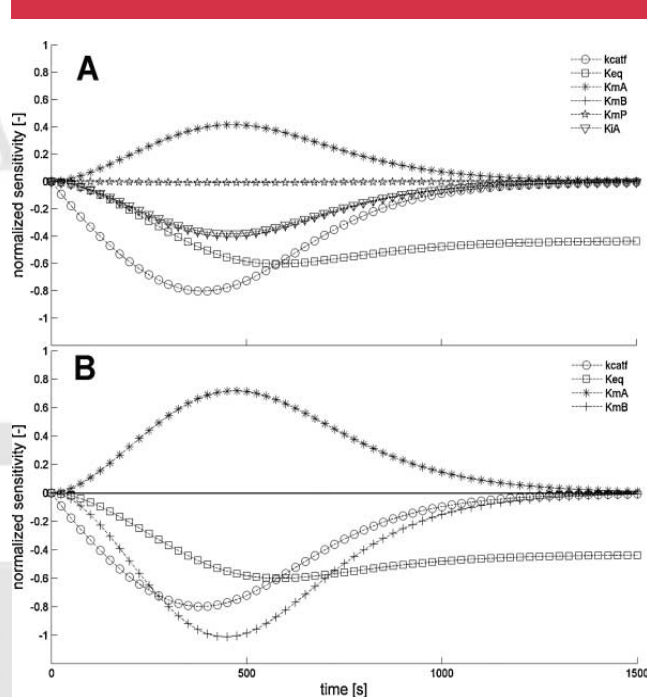


Figure 5. Normalized sensitivities of the model parameters of the derived mechanistic model (A) and of the simplified kinetic model (B).

According to this, k_{catf} is most sensitive at the start of the reaction and, therefore, can be estimated quite precisely. K_{eq} is the only parameter that is sensitive for longer time periods. For this reason, its estimate is also very accurate. K_{mP} turns out to be very insensitive, which explains the high level of uncertainty for this parameter. The parameters K_{iA} and K_{mB} possess almost identical sensitivity curves. Therefore, they are strongly negatively correlated. The sensitivity curve of K_{mA} is inverse to the ones of K_{iA} and K_{mB} , which also causes high correlations to these parameters. For this reason, these kinetic parameters cannot be estimated independently of each other. These findings show that the model comprises too many parameters and has to be revised according to the MEXA methodology.

Model Revision

As shown in Figure 2B, the micro-reaction constants k_1 and k_2 denote the rate constants for the binding of the substrate molecules to the enzyme and the enzyme–substrate complex, respectively. These binding processes can be considered as a sum of three steps. First, the substrate molecules diffuse from the bulk solution to the enzyme surface. Then, the molecules diffuse to the cofactor within the active site. The last step is the binding of the molecules. The first substrate molecule binds to the ylide form of the cofactor and the second to the enamine-carbanion intermediate (Demir et al., 2001b; Fig. 2B). Obviously, the only difference between the first and the second binding substrate is the binding to different forms of the cofactor. The diffusion steps are equally fast since the diffusion coefficients

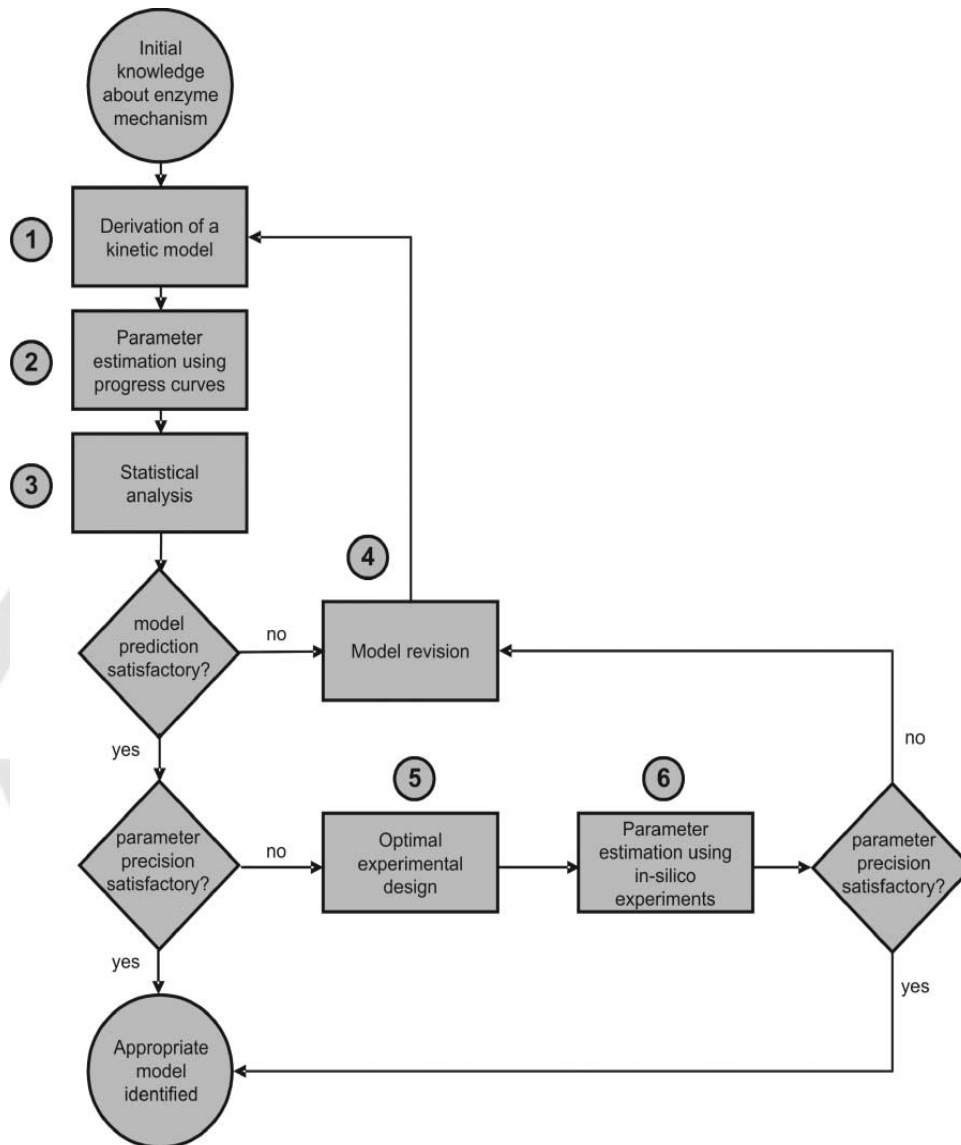


Figure 6. Comparison of the fitted simplified kinetic model with the experimental data using experiments A–I (Table I). The residuals are shown as small inlets.

are the same. Assuming also that the binding step is equally fast, the micro-reaction constants k_1 and k_2 will have the same value. The same assumption is made for the micro-reaction constants k_{-1} and k_{-2} because in this case the same steps occur only in reverse order.

With this assumption only four independent micro-reaction constants remain without neglecting any reaction step. As a result, only four independent parameters can exist. Using the method of Straathof and Heijnen (1996), two more relations between the parameters could be identified (Eqs. 7 and 8), which are added to the kinetic model (Eqs. 2 and 5):

$$K_{mP} = \frac{K_{mB}(K_{mB} - K_{mA})^2 K_{eq}}{2K_{mA}} \quad (7)$$

$$K_{iA} = K_{mB} - K_{mA} \quad (8)$$

Consequently, only four independent parameters, which are listed in Table II, have to be fitted during parameter estimation. It should be noted that the assumptions do not lead to the equality of K_{mA} and K_{mB} .

The modified model has been fitted to the experimental data of the original experiments as described in Table I (experiments A–I). The experimental data together with the fitted progress curves and the residuals are provided in Figure 6. The estimated values are listed in Table III with their 95% confidence regions. Obviously, those parameters can now be estimated much more precisely despite the tight experimental limitations. In Table IVC the correlation matrix is presented. The obtained correlations between the kinetic parameters are satisfactory except for the correlation of K_{mA} and K_{mB} .

With Equations (18)–(21) (Appendix A) the micro-reaction constants were calculated. For the error propagation the Gauss formula cannot be used since there are

correlations between the parameters. Therefore, Equation (9) was used instead (Tellinghuisen, 2001)

$$\sigma_k^2 = J^T C J \quad (9)$$

In Equation (9), σ_k^2 represents the variance in the function for k_i (Eqs. 18–21) containing the model parameters θ_i , whose variance–covariance matrix is $C_{i,j}$ (Table V). The Jacobian matrix J contains the partial derivatives of k_i with respect to the model parameters θ_i :

$$J_i = \frac{\partial k}{\partial \theta_i} \quad (10)$$

Consequently, the standard deviation of a micro-reaction constant σ_k can be calculated with the following equation:

$$\sigma_k = \left(\sum_{i=1}^n \sum_{j=1}^n \left(\frac{\partial k}{\partial \theta_i} \frac{\partial k}{\partial \theta_j} C_{i,j} \right) \right)^{1/2} \quad (11)$$

The calculated values for the micro-reaction constants (Table VI) indicate that the release of the product is rate limiting for the synthesis reaction, while the release of the substrates is rate limiting for the reverse reaction.

Despite the fact that the parameter estimation was much more precise, it can be expected that the estimation would be even more precise if the experimental degrees of freedom would not be so limited. Therefore, new *in silico* experiments (Table I, experiments R–Y) were designed for fitting the simplified model. For the model-based optimal experimental design the same experimental limitations have been used. Once more, the determined optimal experiments have been conducted *in silico*. The simulated data have been disturbed with white noise with a standard deviation of 0.001 mM and used for a new parameter estimation. The results of this step are presented in Tables III and IVD.

Table III implies that the parameter precision can be increased if the model is fitted to experiments with initial product concentrations. The correlations become slightly lower, but the correlation between K_{mA} and K_{mB} still remains high (Table IVD). Considering the definition of these parameters, the remaining high correlation is not surprising since K_{mA} and K_{mB} both contain the term k_3/k_1 (Table II).

For the simplified model, a new dynamic sensitivity analysis was performed, which is depicted in Figure 5B. As

Table V. Variance–covariance matrix of the estimated parameters using the simplified kinetic model.

	k_{catf}	K_{eq}	K_{mA}	K_{mB}
k_{catf}	3.184E–02	1.354E–03	3.534E–04	8.685E–04
K_{eq}		2.011E–04	1.635E–05	3.938E–05
K_{mA}			8.204E–06	2.062E–05
K_{mB}				5.184E–05

Table VI. Calculated micro-reaction constants.

Micro-reaction constant	Calculated value	Standard deviation	Unit
k_1, k_2	3786.90	893.68	$\text{mM}^{-1} \text{s}^{-1}$
k_{-1}, k_{-2}	132.54	14.86	s^{-1}
k_3	45.44	0.35	s^{-1}
k_{-3}	9355.90	2306.60	$\text{mM}^{-1} \text{s}^{-1}$

already observed for the basic model (Fig. 5A), the parameters k_{catf} and K_{eq} can be estimated very precisely since they are very sensitive at the start and at the end of the reaction, respectively. The strong correlation of K_{mA} and K_{mB} is caused by the symmetrical sensitivity curves of these parameters. A possible solution for this could be the modeling of the enzyme reaction directly with micro-reaction constants.

Conclusions

Based on the proposed mechanism for BAL, a kinetic model was derived, which takes all micro-reaction steps into account. In the special case of two identical substrates, two K_m -values exist since one substrate acts as a donor and the other as acceptor. The model contains six independent parameters. Four of them cannot be estimated with sufficient accuracy. There are two reasons for this. First, the experimentally accessible region is rather limited due to the low solubility of the product. This can be investigated with the use of *in silico* experiments, but even under these optimal conditions the model is too complex for estimating all six parameters with high precision. For this reason the model was revised by assuming identical values for k_1 and k_2 , and for k_{-1} and k_{-2} . Therefore, only four independent parameters remain, which can be estimated much more accurately. *in silico* experiments show that under less limited experimental conditions the model parameters can be estimated very precisely. The derived kinetic model could also be applied for other ThDP-dependent enzymes. Most of these enzymes basically follow the same mechanism with the only difference that they are not able to cleave the formed (*R*)-2-hydroxy ketones. This would result in $k_{-3} = 0$.

Nomenclature

BAL	benzaldehyde lyase
BFD	benzoylformate decarboxylase
DMBA	3,5-dimethoxy-benzaldehyde
MEXA	model-based experimental analysis
PDC	pyruvate decarboxylase
ThDP	thiamin diphosphate
TMB	(<i>R</i>)-3,3',5,5'-tetramethoxy-benzoin
A	concentration of first binding substrate (mM)
A_0	initial substrate concentration (mM)

B	concentration of second binding substrate (mM)
C	variance covariance matrix
E	enzyme concentration (mM)
J	Jacobian matrix
k	micro-reaction constant (s^{-1}), ($mM^{-1} s^{-1}$)
k_{catf}	maximum turnover number (s^{-1})
k_{catr}	maximum turnover number for reverse reaction (s^{-1})
K_{eq}	equilibrium constant (mM^{-1})
K_{iA}	inhibition constant for first binding substrate (mM)
K_{iB}	inhibition constant for second binding substrate (mM)
K_{iP}	inhibition constant for product (mM)
K_{mA}	Michaelis constant for first binding substrate (mM)
K_{mB}	Michaelis constant for second binding substrate (mM)
K_{mP}	Michaelis constant for product (mM)
P	product concentration (mM)
P_0	initial product concentration (mM)
S	normalized sensitivity
σ	standard deviation of a micro-reaction constant (s^{-1}), ($s^{-1} mM^{-1}$)
θ	model parameter (s^{-1}), (mM^{-1}), (mM)
v	reaction rate ($mM s^{-1}$)

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Appendix A

Relationships between micro-reaction constants and kinetic parameters of the mechanistic kinetic model:

$$k_1 = \frac{k_{catf}}{K_{mA}} \quad (12)$$

$$k_{-1} = \frac{K_{iA} k_{catf}}{K_{mA}} \quad (13)$$

$$k_2 = \frac{k_{catf}(K_{mP}K_{iA} - K_{mP}K_{mA} + K_{iA}^2 K_{mB}K_{eq})}{K_{mB}(K_{iA}^2 K_{mB}K_{eq} - K_{mP}K_{mA})} \quad (14)$$

$$k_{-2} = \frac{K_{mP}K_{iA}k_{catf}}{K_{iA}^2 K_{mB}K_{eq} - K_{mP}K_{mA}} \quad (15)$$

$$k_3 = k_{catf} \quad (16)$$

$$k_{-3} = \frac{k_{catf}(K_{mP}K_{iA} - K_{mP}K_{mA} + K_{iA}^2 K_{mB}K_{eq})}{K_{mP}K_{iA}^2 K_{mB}K_{eq}} \quad (17)$$

Relationships between micro-reaction constants and kinetic parameters of the simplified kinetic model:

$$k_1 = k_2 = \frac{k_{catf}}{K_{mA}} \quad (18)$$

$$k_{-1} = k_{-2} = \frac{k_{catf}(K_{mB} - K_{mA})}{K_{mA}} \quad (19)$$

$$k_3 = k_{catf} \quad (20)$$

$$k_{-3} = \frac{k_{catf}}{K_{eq}(K_{mB} - K_{mA})^2} \quad (21)$$

Relationships for the dependent model parameters k_{catr} and K_{iP} :

$$k_{catr} = \frac{k_{-1}k_{-2}}{k_{-1} + k_{-2}} = \frac{k_{catf}K_{mP}}{K_{mB}K_{iA}K_{eq}} \quad (22)$$

$$K_{iP} = \frac{k_3}{k_{-3}} = \frac{K_{mP}}{1 - \left(\frac{K_{mA}}{K_{iA}} - 1\right) \frac{K_{mP}}{K_{eq}K_{mB}K_{iA}}} \quad (23)$$

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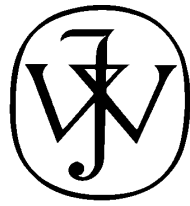
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