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Mechanistic model for prediction of formate dehydrogenase kinetics under industrially relevant conditions

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Abstract

Formate dehydrogenase (FDH) from *Candida boidinii* is an important biocatalyst for regeneration of the cofactor NADH in industrial enzyme catalysed reductions. The mathematical model that is currently applied to predict progress curves during (semi-)batch reactions has been derived from initial rate studies. Here, it is demonstrated that such extrapolation from initial reaction times to performance during a complete batch leads to considerable prediction errors. This observation can be attributed to an invalid simplification applied during the development of the literature models. A novel mechanistic model which describes the course and performance of FDH-catalysed NADH regeneration under industrially relevant process conditions is introduced and evaluated. Based on progress curve instead of initial reaction rate measurements, it was discriminated from a comprehensive set of mechanistic model candidates. For predictions on long time horizons, decomposition of NADH has to be considered. The model accurately describes the regeneration reaction under all conditions, even at high concentrations of the substrate formate and thus is clearly superior to the existing model. As a result, course and performance of NADH regeneration in industrial enzyme catalysed reductions can for the first time be accurately predicted and used to optimise the cost-efficiency of the respective processes.

Keywords

Biocatalysis, formate dehydrogenase, cofactor regeneration, process modelling

INTRODUCTION

In recent years enzyme catalysed reductions of prochiral molecules to chiral compounds have advanced to industrially important synthetic reactions[1]. The applied biocatalysts are oxidoreductases which mediate the transfer of electrons with appropriate donor molecules, most frequently NADH[2]. Thus, a complete synthesis requires stoichiometric amounts of these cofactors unless *in situ* regeneration can be accomplished. As a matter of fact, regeneration is mandatory to render an enzyme catalysed reduction economically feasible[3]. In this context, formate dehydrogenases have become of outstanding importance[3, 4]. This particularly applies to the NADH dependent FDH from *Candida boidinii* (E.C.1.2.1.2) [e.g. 3, 5, 6-8] because of its high operational stability[9].

FDHs catalyse the transfer of electrons to NAD^+ or $NADP^+$ by oxidizing formate to CO_2 (figure 1a). The reaction follows an ordered bi-bi-mechanism consisting of four elementary steps: 1. NAD^+ binds to FDH forming an enzyme-substrate-complex, 2. formate binds to the enzyme-substrate-complex and the resulting construct is rapidly transformed into a ternary product complex, 3. CO_2 is released, and 4. NAD(P)H is set free[10, 11] (figure 1b). The reaction is considered to be practically irreversible as CO_2 shows only low solubility in water and passes into the gaseous phase[12]. Thereby it drives the formation of NAD(P)H to completion.

<Insert figure 1 about here>

The kinetics of this mechanism were described as follows[10],

$$v = \frac{V_{\max} \cdot A \cdot B}{K_{iA} \cdot K_{mB} + K_{mA} \cdot B + K_{mB} \cdot A + A \cdot B} \qquad (1)$$

where v is the reaction rate, V_{max} is the maximum reaction rate, K_i and K_m are kinetic parameters according to Cleland[13], and A and B refer to the molar concentrations of NAD^+

and formate, respectively. The model takes two micro-kinetic steps into account: the binding/dissociation of NAD^+ (Figure 1b, step 1) and the binding/dissociation of formate to/from FDH (Figure 1b, step 2). Since the following two steps (Figure 1b, steps 3 and 4) are neglected in the model, equation (1) is useful under initial rate conditions, but incomplete when considerable amounts of CO_2 and NADH are formed, i.e. under operational conditions.

Another kinetic model, given by equation (2),

$$v = \frac{V_{\max} \cdot A \cdot B}{K_{mA} \cdot K_{mB} + K_{mB} \cdot A + K_{mA} \cdot B + A \cdot B + \frac{K_{mA} \cdot K_{mB}}{K_{iQ}} \cdot Q + \frac{K_{mA}}{\cdot K_{iQ}} \cdot B \cdot Q} \qquad (2)$$

was exlusively established to describe the FDH catalysed reaction during application[14]. Here, Q denotes the molar concentration of NADH. This model lacks K_{iA} and thus implies less kinetic parameters than equation (1). As details on the derivation of the model cannot be found in literature, it remains unclear whether this results from a simplification of (1) or a completely empirical derivation. Anyway, the model has been extensively used to predict the performance of the FDH catalysed cofactor regeneration in coupled enzyme processes[15-23], although the ability of the model to predict more than initial rates has never been systematically investigated. Differences between predictions and the experimentally determined progress of NADH formation by FDH, which were occasionally observed[14], were attributed to the evaluation of initial rates in dilute solutions where substrate and/or product inhibitions might not be detectable.

In this study, a complete mechanistic model for the FDH catalysed reaction is described, evaluated for its ability to predict the reaction course under process conditions, and compared to the performance of the model established by Kula *et al.*[14]. The identification of the FDH kinetics was performed by applying the "incremental method"[24-26] on a complete set of model

candidates as described in detail by Michalik *et al.*[26], using progress curve analysis for discrimination. The parameter confidence was significantly improved by including additional experimental data. The resulting model was used to simulate the course of NADH regeneration at relevant substrate concentrations and compared to experimental data. The same was done with the existing model[14], and discrepancies were thoroughly investigated.

MATERIALS AND METHODS

All chemicals were purchased from Fluka (Buchs, Switzerland) except for NAD^+, NADH, and FDH from *Candida boidinii* (E.C.1.2.1.2) which were obtained from Codexis (Juelich, Germany).

For all experiments, NAD^+ and sodium formate were dissolved in 200 mM sodium phosphate buffer (pH 7.5). The temperature was kept constant at 25°C. The reaction was initiated by adding 50 µL of a 1:100 dilution of the commercial enzyme preparation (104 U/mL; 22 µM) to 950 µL buffer containing the reactants. The concentration of active enzyme was determined by active-site-titration using the triazine dye Procion blue mx-r according to Felber[15]. NADH was assayed at 340 nm using a UV/Vis spectrometer (SpectraMax Plus, Molecular Devices, Sunnywale, California, USA) at a NADH concentration where the relationship to extinction was linear. The path length of cuvettes was 0.5 mm. Molar concentrations of NADH were calculated by application of Lambert-Beer's law with a molar extinction coefficient of 6230 $L \cdot mol^{-1} \cdot cm^{-1}$.

Data for progress curve measurements were obtained with a typical interval of 15 s and a maximum interval of 30 s over a time period of 1 to 20 h. A 3^2 full factorial design was adopted with the degrees of freedom being the initial concentrations of NAD^+ and formate,

respectively. The initial concentration settings were 1 mM, 5 mM, and 10 mM NAD^+, and 50 mM, 500 mM and 1500 mM formate, respectively (table 1). According to Bardow and Marquardt[24], Brendel *et al.*[25], and Michalik *et al.*[26], the models were fitted to the experimental data using the incremental method and in house software (AVT, Chair of Process Engineering, RWTH Aachen University) in a first step and the dynamic parameter estimation algorithm of *gProms* (Version 3.0.2, Process System Enterprise Ltd., London, UK) in a second step. To improve the parameter estimates of the model, two additional experiments were carried out with the initial concentrations of 5 mM NAD^+ / 1 mM formate and 150 mM NAD^+ / 10 mM formate.

<Insert table 1 about here>

The same set of experiments were used to fit the model established by Kula *et al.*[14] (equation 2). All kinetic parameters were estimated using the simulation software *gProms* (Version 3.0.2, Process System Enterprise Ltd., London, UK).

RESULTS AND DISCUSSION

Mechanistic kinetic model. Based on the four micro-kinetic steps underlying the ordered bi-bi-mechanism of FDH-catalysed formate oxidation (figure 1b) a complete set of model candidates describing the reaction was derived. For all micro-kinetic steps irreversibility as well as reversibility was considered and model candidates were developed by combination of all possible set-ups according to the method of King and Altman[27]. Four couples of the resulting mathematical equations were identical, i.e. 12 different model candidates from a possible total of 16 candidates were derived. Some of these candidates were unreasonable in a biological sense. Nonetheless, they were considered for model discrimination in order to

meet the demand for a simple model and to avoid subjective and thus potentially misleading *a priori* assumptions. The model was developed by means of progress curve instead of initial rate measurements, i.e. around 4,800 data points per experiment were available and enabled a very accurate description of the reaction.

The model candidates were discriminated by applying the "incremental method", a novel approach to model identification described by Bardow and Marquardt[24, 28] and refined by Brendel et al.[25]. Basically, this method consists of two steps, both aiming at the identification of optimal model parameters by minimising the error deviation between experimental data and mathematical model predictions. In the first step, the model candidates are evaluated using nonlinear regression. In the second step, the promising candidates are discriminated in a statistically sound manner by dynamic parameter estimation. For details, the reader is referred to the recently published work of Brendel et al.[26]. The macro-kinetic model derived from this approach is given as follows,

$$v = -\frac{dA}{dt} = -\frac{dB}{dt} = \frac{dP}{dt} = \frac{kcat \cdot E \cdot A \cdot B}{K_{iA} \cdot K_{mB} + K_{mB} \cdot A + K_{mA} \cdot B + A \cdot B + \frac{K_{iA} \cdot K_{mB}}{K_{iQ}} \cdot Q + \frac{K_{mA}}{\cdot K_{iQ}} \cdot B \cdot Q} \quad (3)$$

where E refers to the molar concentration of FDH and k_{cat} denotes the turnover number. Retracing the individual reaction steps from this equation shows that all steps except the release of CO_2 from the ternary product complex (figure 1b) are assumed to be reversible. This is in accordance with the currently accepted overall reaction mechanism[10, 11].

Subsequently, for a correct description of the reaction progress over a longer time period, the spontaneous, irreversible decomposition of the cofactor NADH over time was included in the kinetic model. Assuming first-order kinetics as determined by Alivisatos *et al.*[29] and Chenault and Whitesides[30] results in

$$\frac{dQ}{dt} = v - k_{dQ} \cdot Q \qquad (4)$$

where k_{dQ} denotes the rate constant of NADH decomposition.

Prediction of reaction courses. The prediction accuracy with regard to industrial use was evaluated by fitting the model to the progress curves of nine reactions using different initial substrate concentrations. The same was performed with the model established by Kula *et al.*[14], enabling a comparison between estimates from the novel mechanistic model and the model used in previous studies[15-23]. Both models are applied by considering the spontaneous decomposition of NADH according to equation 4.

Parameter estimation was performed using starting values from literature (table II) along with 200 randomly chosen parameters in order to decrease the risk of getting stuck in local optima. The experimental design (table I) for progress curve analysis suggested an excess of formate and low concentrations of NAD^+ to match economically relevant conditions. Nevertheless, two additional experiments were conducted at excess initial NAD^+ (100 mM/10 mM) and formate close to K_m (5 mM/1 mM) (data not shown) to optimise the confidence intervals of K_{mB}. The values estimated for the kinetic parameters using the novel mechanistic kinetic model and the model established by Kula *et al.*[14], respectively, are summarised in table 2. The small standard deviations suggest that all values are statistically significant and estimated with good accuracy.

<Insert table 2 about here>

The results of the investigation demonstrate the excellent ability of the new mechanistic kinetic model to predict the course of NADH regeneration by FDH over long periods of time at all substrate concentrations (figure 2). This implies that the model gives a mechanistically reasonable representation of the reaction. A high specificity of the enzyme for both substrates

occurs, and in accordance with published data, NADH exhibits a strong competitive inhibition with respect to NAD^+. Reaction rates increase with increasing concentrations of formate, whereas the concentration of NAD^+ mainly influences the time until the reaction is completed. The latter corresponds to the comparably low concentrations of NAD^+ in the investigated reaction set-ups.

The negative slope of some progress curves at later reaction times can be explained by spontaneous decomposition of NADH. From the observed decay, the rate constant of NADH decomposition k_{dQ} (equation 4) was calculated to be $9.59 \cdot 10^{-6}$ s^{-1} corresponding to a half life-time of NADH of about 20 h. This is in good accordance with the findings of Chenault and Whitesides[30] who reported a half life-time of 27 h. The deviation can be explained by the use of different buffer systems. The experiments reported here were performed at a 0.5 units higher pH and a twice as high buffer capacity. According to Alivisatos *et al.*[29], the resulting increase of $H_2PO_4^-$-concentration causes a decrease of NADH stability.

In contrast to the novel mechanistic kinetic model, the model established by Kula *et al.*[14] gave accurate predictions for NADH formation only when the initial concentration of formate was low and/or the reaction time was short (figure 2). Thus, the application to predictions under process conditions is not reasonable, as formate is usually required at high concentrations, while NAD^+ is regenerated and kept low.

The parameters estimated with the two models (equations 2 and 3) deviate particularly in K_{mA} and k_{cat}. Both are one to two magnitudes higher when estimated with the model by Kula *et al.*[14]. However, K_{mA} is more in the range of reported values when estimated with the novel mechanistic kinetic model. Furthermore, measurements at saturation of formate or NAD^+ and excess amounts of the respective reaction partner (data not shown) indicate that the estimation of k_{cat} with the novel mechanistic kinetic model (0.18 s^{-1}) is correct, while the

model by Kula *et al.*[14] leads to an overestimation (1.35 s^{-1}). The observations suggest that K_{mA} and k_{cat} in the model by Kula *et al.*[14] are fits without physical meaning.

It was found that the model of Kula *et al.*[14] can be derived from the novel mechanistic model by assuming that K_{mA} equals K_{iA}, known as Michaelis-Menten-constraint[31]. This effect can therefore be considered responsible for the low prediction accuracy, and is in accordance to the findings of Biselli *et al.*[32] and Frey and Hegemann[2] that for many enzyme catalysed reactions the Michaelis-Menten-constraint is not valid.

A comparison of the parameter values obtained from the model of Kula *et al.*[14] and those reported in literature reveals significant deviations, though all reported data were derived by means of the same model. This can be explained by the fact, that all published parameters were determined from initial rate measurements instead of reaction courses. Furthermore, these parameters cannot be used to predict NADH regeneration under process conditions.

CONCLUSIONS

With the here described mechanistic kinetic model, a description for the kinetics of FDH from *C. boidinii* has been found which applies to conditions under industrial use. Thus, course and performance of NADH regeneration in industrial enzyme catalysed reductions can for the first time be accurately predicted and used to optimise the cost-efficiency of the respective processes.

In the systematic model development approach, the use of the “incremental method” not only simplified model identification and parameter estimation, but resulted in an improved model structure which is obviously closer to the true reaction mechanism. A simplification of the novel model results in the literature model; hence, the higher degree of generality of the novel

model is demonstrated. According to our findings, the more general novel model is of an apparently better predictive quality under process conditions than the simplified literature model. Because of the significant uncertainty in the application and interpretation of initial rate measurements, progress curves should be preferred. Furthermore, only progress curve data cover the concentration range at reasonable experimental effort and hence facilitate the prediction not only of continuous but also of batch reactions. Though progress curve analysis may still be considered difficult and time-consuming to apply by experimenters, readily available software tools, the increasing number of published success stories and the significant reduction of the experimental effort will pave the way to a wide-spread use in biotechnology research and practice.

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List of figure legends:

Fig. 1 a) FDH catalysed reaction including generation of NAD(P)H from $NAD(P)^+$. b) Micro reaction steps (r_i) of the FDH catalysed reaction including NADH decomposition. Substrate-enzyme and product-enzyme complexes are put in parantheses.

Fig. 2: Prediction accuracy of novel mechanistic and literature model. Experiments were conducted according to a 3^2 design. ——— measured data; — — — predictions of mechanistic model; – – – – predictions of literature model.

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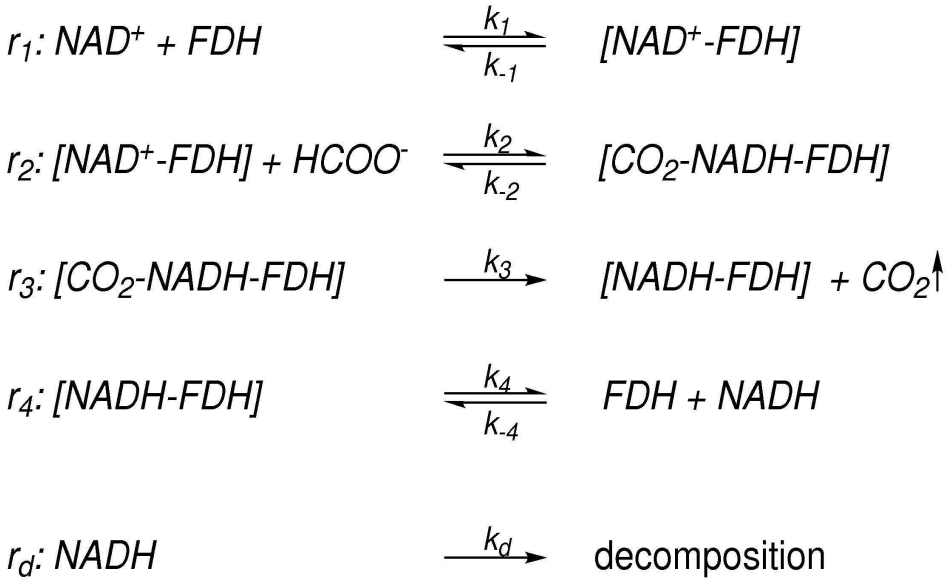

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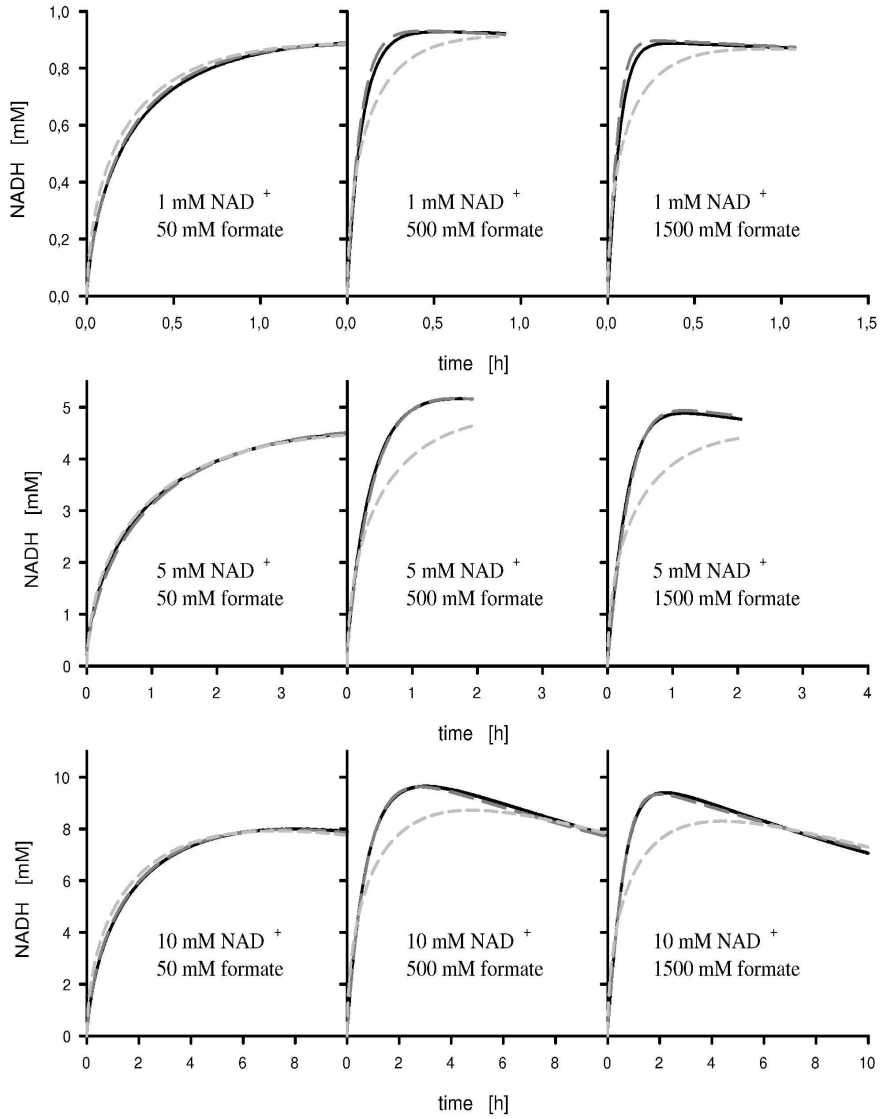

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Tables:

Table 1: Experimental design for progress curve measurements. Experiments A to I were planned in a 3^2 factorial design.

		Formate [mM]		
		50	500	1500
NAD^+ [mM]	1	A	B	C
	5	D	E	F
	10	G	H	I

Table 2: Optimal kinetic parameters derived from progress curve analysis for novel mechanistic and literature model along with a summary of available literature values determined by initial rate measurements. Assay conditions are given. Unavailable informations are denoted with -.

K_{mA} [µM]	K_{mB} [mM]	K_{iQ} [µM]	K_{iA} [mM]	T [°C]	pH	Reference
38,39 ± 0,09	0,472 ± 0,007	117,5 ± 0,04	78,14 ± 0,08	25	7,5	Novel mechanistic model
1702 ± 34	(63,0 ± 0,2)·10^3	68,3 ± 1,2	-	25	7,5	Literature model
39	12.8	289	-	30	8.0	[16]
45	6.0	-	-	30	-	[15]
40	2.4	-	-	25	-	[33]
45	5.6	17	-	30	7.5	[34]
39	8.70	30	-	-	8.0	[35]
70	29.34	90	-	30	8.0	[22]
300	94.5	150	-	40	8.0	[23]
32	1.76	6.4	-	-	8.0	[11]
100	16	30	-	30	8.0	[10]
90	13	-	-	30	7.5	[12]
100	1.5	-	-	-	7.5	[36]

Appendix: Definitions of model parameters for mechanistic kinetic model and literature model.

Parameter	Unit	Mechnistic model	Literature model	Biological interpretation
k_{cat}	s-1	$\frac{k_3 \cdot k_4}{k_3 + k_4}$	$\frac{k_3 \cdot k_4}{k_3 + k_4}$	maximum turnover number
K_{mA}	mM	$\frac{k_3 \cdot k_4}{k_1 \cdot (k_3 + k_4)}$	$\frac{k_3 \cdot k_4}{k_1 \cdot (k_3 + k_4)}$	binding constant of A to E
K_{iA}	mM	$\frac{k_{-1}}{k_1}$	$\frac{k_3 \cdot k_4}{k_1 \cdot (k_3 + k_4)}$	dissociation constant of EA
K_{mB}	mM	$\frac{(k_{-2} + k_3) \cdot k_4}{k_2 \cdot (k_3 + k_4)}$	$\frac{(k_{-2} + k_3) \cdot k_4}{k_2 \cdot (k_3 + k_4)}$	binding constant of B to EA
K_{iQ}	mM	$\frac{k_4}{k_{-4}}$	$\frac{k_4}{k_{-4}}$	dissociation constant of EQ