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PAPER

Formate dehydrogenase - a biocatalyst with novel applications in organic chemistry

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In the field of industrial biocatalysis, formate dehydrogenase (FDH) is well established, in particular for its broad application in cofactor regeneration. Further applications have been limited by the enzyme's narrow range of substrates. These restrictions have been overcome now by the finding, that the enzyme is capable of selectively cleaving formic acid esters to the respective alcohol. Five homologous alkyl formates and phenyl formate as an aromatic ester were converted quantitatively by FDH from *Candida boidinii* in a batch reaction within 3 to 5 h. The substrates were turned irreversibly into carbon dioxide and the respective alcohol through hydride abstraction from the formyl group with full conversion. The mechanism shows parallels to hydrolysis reactions of the $A_{AC}1$-type. K_M-values and reactions rates of the tested formic acid esters display a tendency to higher conversion rates with increasing chain length. FDH emerged to be a superior deformylation catalyst compared to hydrolases as well as classical catalysts, as was shown by the selective deformylation of 1-acetoxy-4-formoxy butane (92%) and 1,3-bis(3-formoxypropyl)tetramethyldisiloxane. In particular its capability to distinguish between formic acid esters and non-formic acid esters renders the method particularly suitable for protective group chemistry. Furthermore the completeness of deformylation allows for converting substrates highly incompatible with aqueous media like siloxanes within a few hours.

Introduction

Formate dehydrogenase (FDH, EC1.2.1.2) is well-established as a redox enzyme for the regeneration of NADH in cofactor-dependent biocatalysis.[1,2] In industrial processes the enzyme is involved in the large-scale production of *tert*-L-leucine[3,4] for instance. A significant advantage arising from FDH-supported processes is the circumstance of the oxidation product CO_2 being chemically inert, while the thermodynamic equilibrium is completely shifted to the product through release of the gas.[5] In fact it is irreversibility which renders this reaction indispensable in industrial biocatalysis. Typically enzymatic reactions are characterised by being reversible, thus in most instances only incomplete conversion can be achieved. As a consequence space-time yields are unfavourable and the processes are economically less attractive. This holds true particularly for hydrolase mediated reactions where considerable effort is undertaken in order to reach full conversion as this is done for instance, in deracemisation reactions with vinyl acetate. The analogy of forming a gaseous product which irreversibly drives the reaction to full conversion prompted us to investigate the FDH as a potential candidate for the irreversible cleavage of formic acid esters–thus an oxidative alternative to classical enzyme-driven ester hydrolysis.

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There exists a series of different methodologies to synthesise formic acid esters starting from primary and secondary alcohols, phenols or bromides (Table 1). Their deprotection can be achieved by chemical or enzymatic methods, what in the latter case is usually done by hydrolase action on the substrate.[6,7] This approach allows mild reaction conditions, but suffers from the abovementioned drawbacks of equilibrium-mediated re-esterification. Moreover, hydrolases will not differentiate between acylated residues in general and formylated moieties in special, which means that a selective deprotection of differently masked functional groups is not possible as shown in Fig. 1. Deprotection by chemical methods has been thoroughly investigated in organic chemistry as well.[8]

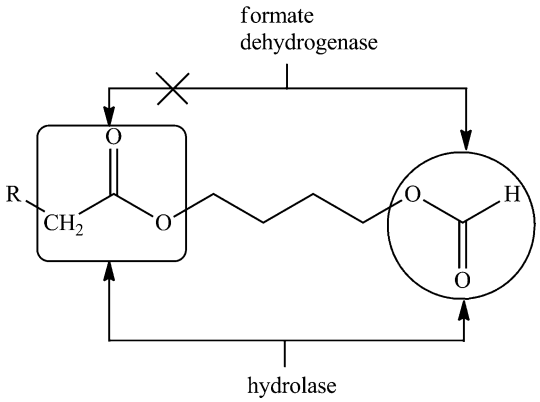
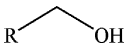
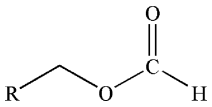
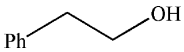
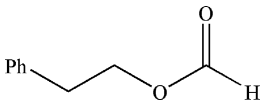
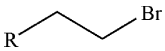
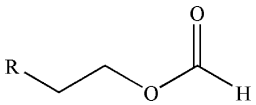
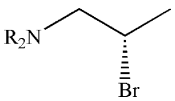
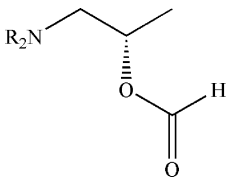


Fig. 1 Chemoselectivity of hydrolase *vs.* FDH-mediated ester cleavage.

Table 1 Methods for formylation of selected substrates

Functionality	Substrate	Conditions/ Catalyst	Product	Ref.
Primary alcohols		Cyanuric chloride		9,10
		Trichloro-1,3,5-triazine		
Primary bromides				
Secondary bromides		DMF[a], DIPEA[a] as base PPh_3/CBr_4[a] in methyl/ethyl formate		11,12

[a] DMF = *N*,*N*-Dimethylformamide, DIPEA = *N*,*N*-Diisopropylethylamine, PPh_3/CBr_4=Triphenylphosphine/Tetrabromomethane

However, these approaches suffer from rather harsh reaction conditions and poor selectivity, thus displaying a lack of methodology in particular for the modification of pre-functionalised complex substrates as *e.g.* in pharmaceutical synthesis. It is for these reasons why FDH appeared as a promising candidate to overcome these shortcomings.

The catalytic mechanism of hydride transfer from formate to NAD^+ is well examined and lots of theoretical and practical investigations have been done thus far.[13–15] If hydride abstraction were realisable with the neutral, protonated species, there would be no reason why the enzyme should not be capable of also cleaving formic acid esters. This oxidation reaction would yield CO_2 and the respective alcohol. It would almost indistinguishably resemble of a conventional hydrolase-mediated ester cleavage accompanied by a FDH-mediated shift of the equilibrium to the product through CO_2 release. Yet, in this case no differentiation between formyl and other acyl groups would be observable (Fig. 1).

Whether or not the concept holds true what can be derived from only scarce literature reports which between themselves diverge in their information on substrate specificity of FDH. An activity towards esters and thioesters of formic acid has been discussed for enzyme preparations from bacteria (*e.g. Pseudomonas* sp. 101,[16] *Achromobacter parvulus*[17]) and from higher plants (*e.g.* pea seeds of *Pisum sativum*[18]). An activity towards the thioester *S*-formylglutathione (GSF) which has been described for the methylotrophic yeasts *Hansenula polymorpha* and *Pichia pinus*[19] as well as *Candida boidinii*[20] and *Kloeckera* sp. 2201[21] has been attributed to *S*-formylglutathione hydrolase.[22,23] In fact the abovementioned enzymatic activity of pea seed extracts towards formic acid derivatives was shown to result from exactly this hydrolase[18] which is able to hydrolyse GSF even in the absence of a cofactor. Any activity whatsoever of FDH towards formic acid esters has not been described thus far, with the exception of one single report on ethyl formate being hydrolysed in aqueous medium in the presence of FDH.[18] However this reaction has been attributed to hydrolase impurities of the FDH used. In addition there exists no further information on autohydrolysis. Thus, from literature no reliable information could be derived whether or not FDH is capable of cleaving formic acid esters.

For mechanistic considerations we initially discussed a working hypothesis based on undissociated formic acid, as this is usually done in hydrogen transfer catalysis. To be precise, the FDH-reaction yields a proton and a hydride ion under evolvement of carbon dioxide.[22]

According to this initial postulate, hydride transfer to NAD^+ is supported by concomitant nucleophilic attack of water at the acid proton. This concept can easily be translated into the cleavage of formic acid esters, where the attack of water takes place at the C-1 atom of the alkyl- or aryl residue. This analogy can be expressed as given in Fig. 2. From protein structure data[23,24] the alignment of a formic acid ester and its stabilisation through the positively charged side chains of asparagine and arginine is conceivable to proceed accordingly to the binding of formate which is the natural substrate. The alkyl- or aryl residue of the substrate would find sufficient space in the substrate binding channel. Consequently, the reaction would yield $NADH/H^+$, CO_2 and the respective alcohol (Fig. 2).

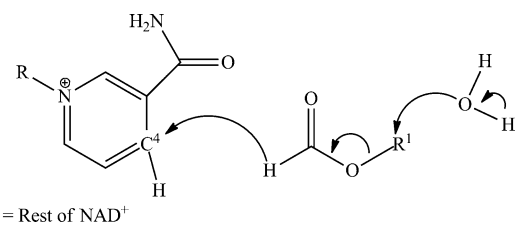


Fig. 2 Initial working hypothesis on the catalytic mechanism of hydride transfer from formic acid and formic acid esters, respectively, to NAD^+.

Hence, the FDH-catalysed dehydrogenation of formic acid esters would turn out to be a potent and selective method for the irreversible deprotection of formyl protected alkyl and aryl alcohols. The proposed mechanism, an initial working hypothesis only, applies for formic acid esters only and is not suitable for other organic acid derivatives. It was for this reason why we undertook a detailed study on the FDH-mediated cleavage of formic acid esters.

Results and discussion

Formic acid ester cleavage by FDH

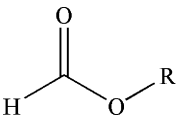
In order to validate the concept of FDH catalysed cleavage of formic acid esters, initial studies were undertaken with **2** as an exemplary substrate. The chromatogram in Fig. 3 shows the progress of the reaction within four hours. As can be seen, the concept holds true: there is a clear consumption of **2** and ethanol is being produced. What can be retrieved from Fig. 3 in addition is the missing of formic acid even in trace amounts. From these results can be taken that there is an oxidative but not hydrolytic cleavage of the ester bond.

After the concept of FDH-mediated cleavage of formic acid esters had been proven true with the example of **2**, also the homologues **1** and **3–6** were investigated. Since the initial working hypothesis discussed a potential nucleophilic attack of water at the carbinol C, submission of an aryl formate to FDH action would provide crucial data for elucidating the mechanism of FDH-mediated cleavage of formates.

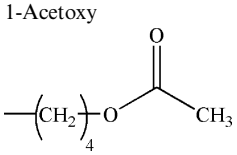
The results of the FDH-catalysed quantitative ester cleavage are shown in Fig. 4. As reaction products an alcohol and CO_2 are evolved. As can be seen from Table 2 higher conversion rates were revealed with increasing chain length. The methyl ester was converted nearly half as slow as the butyl ester **4**. This implies a higher affinity of long-chain formates within the catalytic centre of FDH. The solvolysis of **1–6** by water was almost linear in a range between 0 . . . 50% and fast hydrolysis was observed for **5**.

The hydrolysis rate of **5** by water at neutral pH value leads to an exponential decrease of the content to 14% after 180 min. This was in evident contrast to the linear hydrolysis rate by aliphatic formates. Among the tested formate esters the mixed one **6** displayed the fastest reaction within the first 60 min as depicted in Fig. 4F. FDH was shown to catalyse the cleavage of the formate group with high specificity while the acetate group remained unaffected. Hydrolysis contributed to total conversion in an extent observed for **1–4**. Thus even a rather complex disubstituted ester, 1-acetoxy-4-formoxy butane (**6**), was shown to be a substrate for FDH. This result gives rise to a new perspective on the role of FDH in protection chemistry of alcohols to catalyse the deprotection step of formate esters under mild conditions. Needless to mention that potential hydrolase impurity interfering with the FDH-mediated reaction had been ruled out to occur (*vide infra*).

Table 2 Conversions of formates **1** – **6** by FDH-catalysed cleavage after a reaction time of 3 h. The data are corrected by the amount of water assisted hydrolysis of formates



	R	Conversion in (%)
1	Methyl	23 ± 1,5
2	Ethyl	26 ± 4,7
3	n-Propyl	39 ± 3,0
4	n-Butyl	41 ± 3,7
5	Phenyl	86 ± 4,4
6	1-Acetoxy	59 ± 2,4



The kinetic data K_M and v_{max} were determined for all alkyl formates **1–3** as demonstrated in Table 3.

Table 3 Kinetic constants for FDH-mediated formate ester cleavage

Substrate	v_{max} (U/mL)	K_M (mM)
Sodium formate	49,7 ± 1,8	4,4 ± 0,48
1	10,3 ± 0,7	2,9 ± 0,28
2	12,8 ± 0,5	4,1 ± 0,31
3	15,9 ± 1,4	4,7 ± 0,41
5	21,7 ± 0,60	1,7 ± 0,06

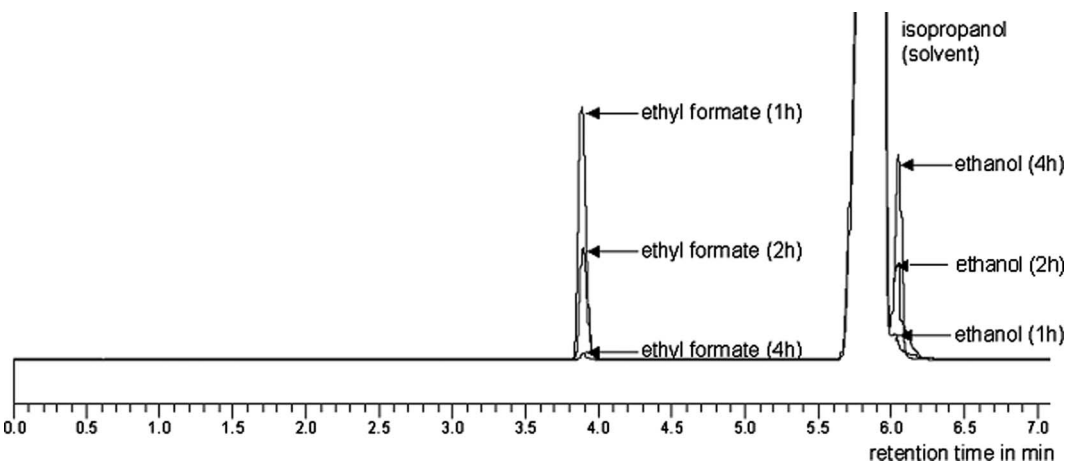


Fig. 3 FDH-catalysed cleavage of **2**.

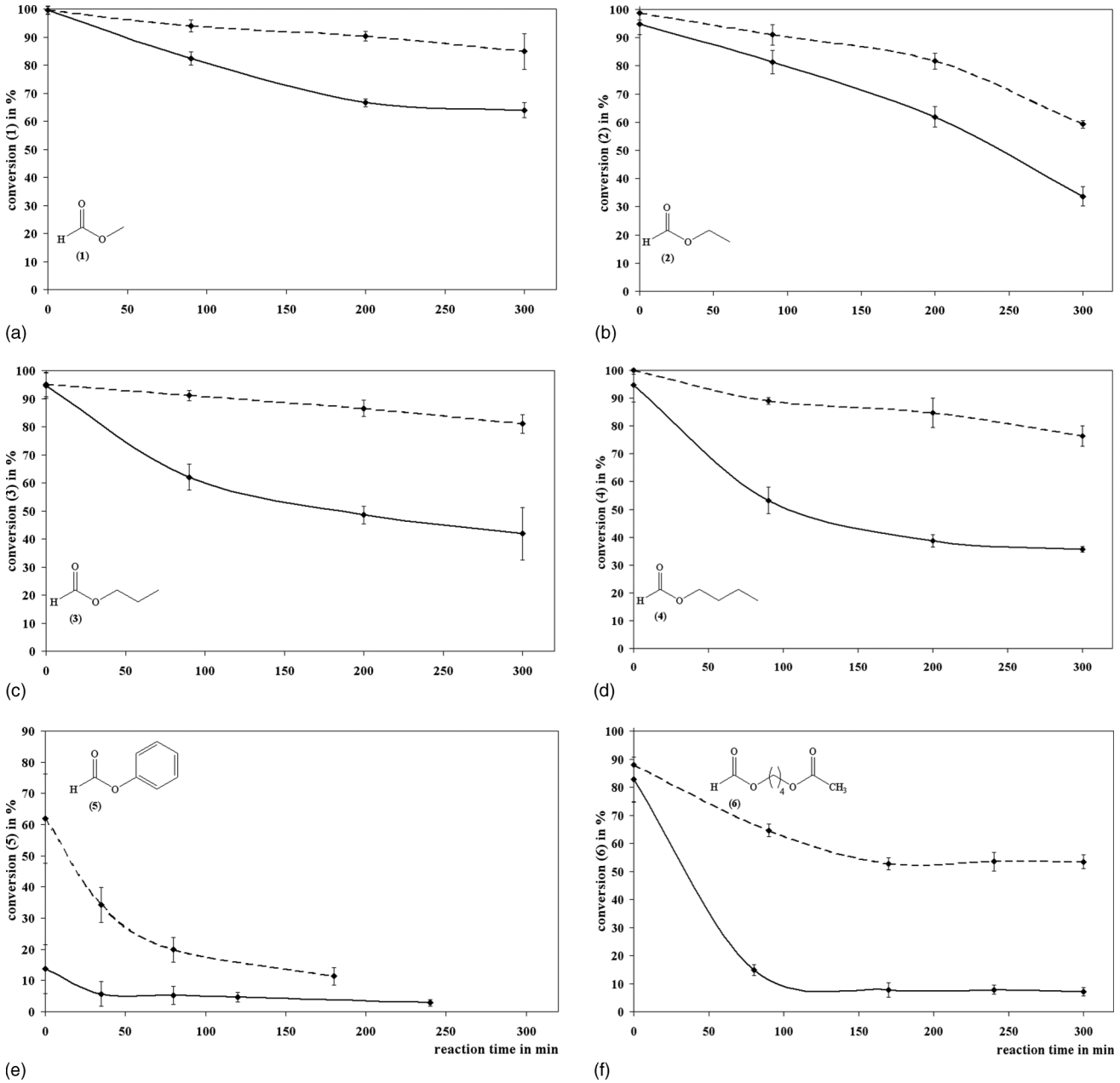


Fig. 4 Cleavage of aliphatic and aromatic formic acid esters **1–6** by FDH. The dashed lines illustrate the hydrolysis rate of formic acid esters by water.

What can be retrieved from Table 3 is the small difference between the v_{max}-values of "natural" substrate sodium formate and the non-classical ester substrates which differ by no more than a factor of ~2 . . . 3. However, it was noticeable that the maximum reaction rate for phenyl formate **5** was twice as high as that for methyl formate **1**. And the K_M-value of **5** is even lower than that of the natural substrate sodium formate. The K_M-value determined for the latter is in line with literature sources which report the value to range from 1,7 to 16 mM.[13,20,25–27] The experimentally determined K_M-values of the alkyl esters (Table 3) imply a correlation between chain length, *i.e.* hydrophobicity, and K_M. It is for this reason why a higher K_M-value for **5** would have been only too plausible, yet the value is the lowest one among all. This reaction behaviour is proposed to be understood in terms of secondary stabilisation effects within the active site of the enzyme inferred by the aromaticity of the phenyl ring.

The active site is characterised by several hydrophobic clusters, among which there are two aromatic amino acids (Phe98, His332) with which aromatic substrates may undergo π–π–interactions. There is further stabilisation by hydrophobic interactions with Pro97 and Val309 (Fig. 5).[24] These secondary interactions may be suited to promote the rate of oxidative cleavage of aromatic formates. On the other hand Pro97 and Val309 are not in direct proximity to the far smaller alkyl substrates **1–4** which therefore experience only weaker stabilising effects. Moreover, it appears that the chain length dependent increasing K_M-values are the outcome of stabilising effects within the active centre. From a mechanistic point of view it cannot be excluded that also leaving

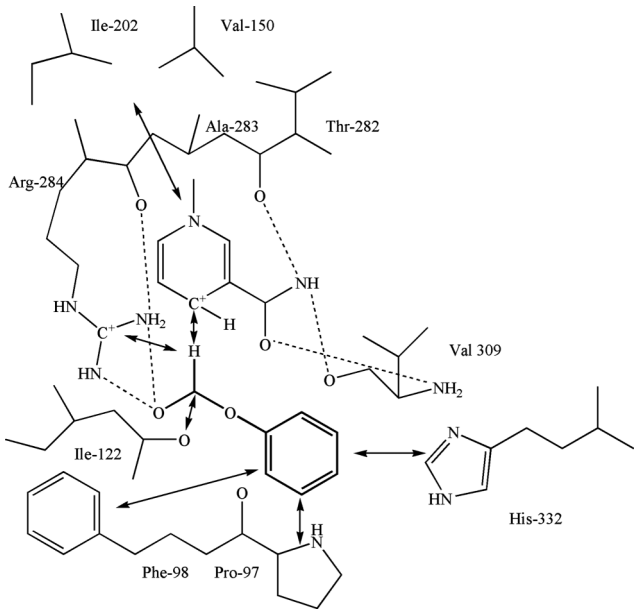


Fig. 5 Hydrophobic areas around the active site of FDH to stabilise aromatic and long-chain formic acid esters as demonstrated for **5**. Arrows illustrate π–π–interactions and dashed lines represent hydrogen bond linkages.

group properties contribute to the susceptibility of a formic acid ester towards cleavage by FDH. These issues require further clarification which is matter of forthcoming studies.

Non-enzymatic cleavage

The enzymatic cleavage of formates is accompanied by non-enzymatic solvolysis through water, resulting in a promotion of reaction rate and conversion, respectively (Table 4). Non-enzymatic hydrolysis of alkyl formates contributes up to 14% of the conversion after 240 min, while more than 35% of **5** were hydrolysed already after 70 min. (Fig. 4). This effect is interpreted as an outcome of the good leaving group properties of phenolate, thus rendering **5** a good substrate for nucleophilic attack of water (pH = 7.5). Formic acid generated by non-enzymatic hydrolysis was deprotonated by the buffer medium in order to exclude side effects caused by acid catalysis.[28] Under the conditions of FDH-mediated cleavage solvolytically produced formic acid was converted into CO_2 instantly and did therefore not exert catalytic effects on formic acid ester hydrolysis. In fact there was no free

Table 4 Degree of hydrolysis of formic acid esters by water

Formate	Degree of hydrolysis in (%)
1	15,2 ± 0,40
2	40,7 ± 0,72
3	19,0 ± 0,38
4	23,6 ± 0,80
5	88,7 ± 10,6
6	46,6 ± 1,50

formic acid or formate detectable in presence of FDH. On the other hand the instantaneous *in situ* removal of formic acid released from the ester through the enzymatic action of FDH on first sight may appear sufficient to explain the observed effects (Fig. 4). However this approach is not in line with the time dependent product formation, and it is by no means suitable to explain the formation of mixed carbonates (*vide infra*). It was these observations that prompted us to check for underlying hydrolase activity.

Potential contribution of competing enzymatic activity

In order to ensure that the observed hydrolysis was not the outcome of hydrolase impurities acting on ester bonds (see Fig. 6), a differentiation assay with *p*-nitrophenyl acetate[40] was performed. Any hydrolase present in the enzyme preparation would result in a conversion of the substrate to *p*-nitrophenol. The differentiation assay was performed with and without addition of a protease inhibitor cocktail, whereas in both cases blanks were determined in analogous manner with buffer instead of enzyme solution. No hydrolase activity was detected in any of the samples, so any impurities could be excluded. Adulterant effects through non-enzymatic reactions of NAD^+ with phosphate ions become effective only at concentrations ≥1,5 M[29] for which reason no such effects were effective to a considerable extent in the 0,1 M phosphate buffer used. There were no indications for a non-enzymatic reduction of the cofactor. Consequently any increase in absorption detected by photometric measurements was attributable to FDH-activity.

Our hypothesis of FDH playing the active part in cleaving carbonic acid esters experienced further corroboration by the observation of no homologous aliphatic ester ($C_2 \ldots C_6$) being cleaved through the action of FDH. In fact formic acid esters as the sole species bearing an abstractable hydride-H are the only entities suitable for enzymatic attack by FDH.

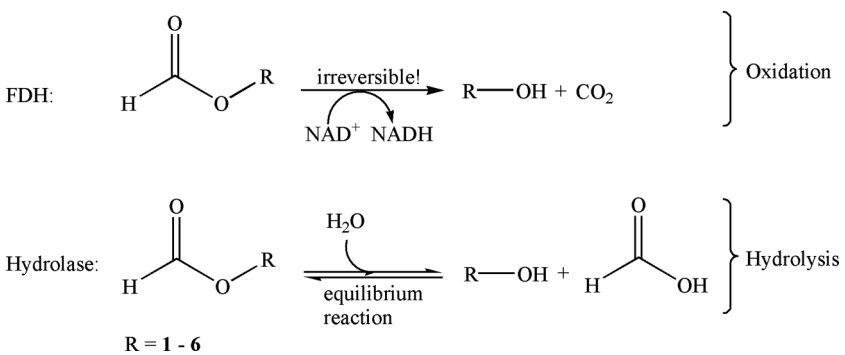


Fig. 6 Formic acid ester cleavage by FDH and hydrolase.

Table 5 Alcohol tolerance of FDH

Alcohol	IC_{50} (vol.-%)
Methanol	10,2 ± 0,9
Ethanol	4,8 ± 0,3
n-Propanol	2,5 ± 0,1
Isopropanol	4,7 ± 0,3
n-Butanol	1,4 ± 0,1
Phenol	0,32 ± 0,01
1-Acetoxy-butane-4-ol	15,6 ± 1,0

Effects of released alcohol on enzyme activity

The alcohols released from their formic acid esters through the action of FDH were tested for potential effects on FDH-CB activity in the range of 0 . . . 45 vol.-% (Table 5). For all tested species a notable tolerance was observed *i.e.* there is no significant enzyme deactivation to be expected at typical working contents of 0.50 . . . 1.29% (v/v). The only exception is made by phenol which is known as an effective protein denaturing agent and potential inhibitor for dehydrogenases.[30,31] Here concentrations exceeding 0.89% (v/v) resulted in significant loss of activity, and the enzyme was fully inactive at c > 0.9% (v/v). On first sight these results may appear to contradict the fast cleavage rates observed for **5** (Table 2, entry 5). For the reasons discussed above, **5** exhibits excellent substrate properties through stabilising interactions with hydrophobic amino acid side chains in the active site of the enzyme. On the other hand the released carbinol, phenol, is a potent enzyme deactivator. Taken together enzyme deactivation through the action of phenol which becomes more effective with increasing phenol concentration competes with fast substrate conversion. Obviously the protein deactivating effect does not become significantly effective within the time frame of ester cleavage.

Mechanistic considerations

While the mechanism of FDH-mediated formate oxidation is well explored,[32] little is known about the mechanism of FDH-mediated ester cleavage. We therefore aimed at elucidating the process of the latter on a molecular level.

Because experimental data obtained in the course of these investigations did not provide support for the working hypothesis initially discussed and illustrated in Fig. 2, we now discuss another mechanism which is well suited to explain the experimental observations made. The proposed mechanism bears analogies to an $A_{AC}1$-type of ester hydrolysis as introduced by Ingold[33] (Fig. 7, pathway 2).

One of the most important features of the $A_{AC}1$-mechanism is the involvement of carbocations doubly bound to oxygen (**a**, **a′**, **c**). The latter is known to withdraw electron density (-I-effect) and to undergo resonance with carbocations (+M-effect) what is a common motive in a multitude of reactions and constitutes a motive well-known from biochemical reactions, *e.g.* in glycogen metabolism.[34] As can be seen in Fig. 7, the electrophilic carbocation **c** reacts with water to form a protonated carboxylic acid, here formic acid (**d**). The deprotonation of the latter yields the organic acid (**e**) in its neutral, undissociated form.

This underlying principle was supposed to apply to the FDH-catalysed oxidative ester cleavage as well (Fig. 7, pathway 1). Hydride abstraction through the enzyme would yield a resonance stabilised cationic entity (**A**, **A′**) which is turned into a carbonic acid monoester (**B**) in analogy to the $A_{AC}1$-pathway (Fig. 7, pathway 2). What distinguishes the FDH-catalysed reaction from the $A_{AC}1$-pathway is the special spatial arrangement of substituents at the carbonyl moiety. A transitional five-membered ring formation, including the non-binding orbitals of ester oxygen, favours the transfer of acid hydrogen from the formyl residue to the latter. Thereby an electronically unstable betaine (**C**) is produced which decomposes under release of carbon dioxide and the respective alcohol (**D**). The process is favoured both entropically and electronically by extrusion of a gaseous neutral compound.

According to this new mechanistic model, three compounds are to be expected in the reaction mixture. Firstly, the carbonic acid monoester (**B**) has to be detectable, at least in trace amounts. Dialkyl/diaryl carbonate (**E**) is formed by reaction of the carbocation (**A**, **A′**) with alcohol (**D**) which is more nucleophilic than water, and finally isopropyl alkyl/aryl carbonate (**F**), where the solvent isopropanol traps the carbocation (see Fig. 8).

Fig. 9 represents the gas chromatogram of FDH-catalysed cleavage of **1** in which a peak at 4,3 min was determined by MS analysis corresponding to the mixed carbonate methyl isopropyl carbonate with characteristic fragments at $m/z = 59$. At the same time, the intermediary formation of formic acid or formate had

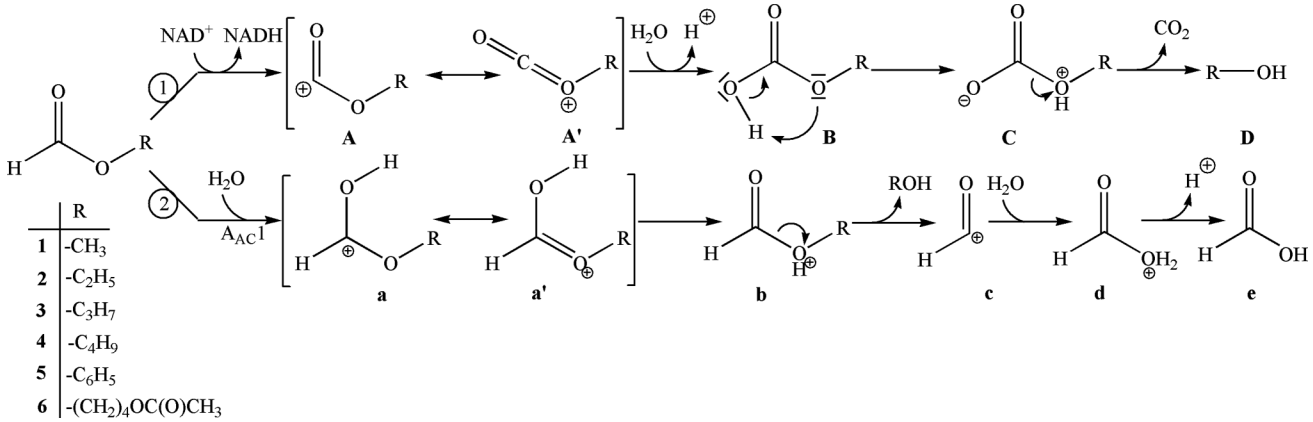


Fig. 7 Pathway 1 - proposed mechanism for FDH-catalysed cleavage of alkyl and aryl formates, pathway 2 - $A_{AC}1$-type of ester hydrolysis.

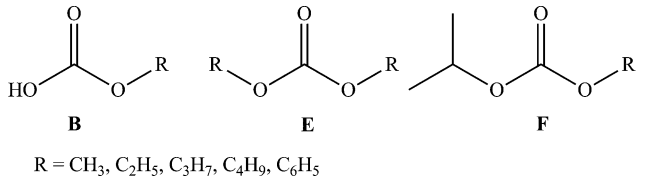


Fig. 8 Formation of species B, E and F confirms the proposed mechanistic model.

to be excluded. Furthermore, release of carbon dioxide had to be demonstrated.

Application of FDH mediated ester cleavage in preparative and protective group chemistry

In the course of the reaction intermediate carbonic acid monoesters of type **B** are formed. These species are relatively unstable, and by releasing carbon dioxide, the reaction equilibrium is irreversibly shifted towards the free alcohol. This feature is of invaluable use for enzyme-catalysed hydrolytic reactions, in particular where these proceed within a time frame that allows the equilibrium between hydrolysis and re-esterification to establish. Under these conditions full conversion cannot be achieved, and the yields obtained will be low. This applies particularly to low substrate concentrations. Phase interfacial phenomena, poor substrate solubilities or low functionalisation densities are possible drawbacks that can be overcome by using the FDH reaction.

However, for all technical applications of FDH a cost-effective cofactor regeneration system is a critical aspect. A regeneration process comprising NADH-dependent reduction of acetone by alcohol dehydrogenase from *Candida boidinii* (ADH-CB) is well-established for industrial use (see Fig. 10).[35,36] Isopropanol which

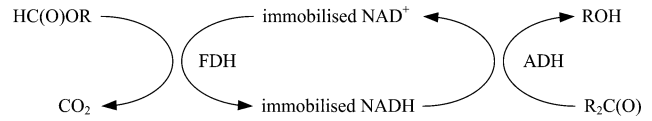


Fig. 10 Regeneration of NAD^+ by ADH-catalysed reduction of acetone to isopropanol.

is the product of the regeneration system, was shown to not interfere with the FDH-mediated ester cleavage. The same applies for the sacrificial substrate acetone on FDH. It cannot be excluded however that in the presence of a dehydrogenase and NAD^+ free alcohol groups of the product are subject to enzymatic oxidation. On the contrary no such process has been observed to take place with ADH-CB so far. It was shown in these investigations that up to 40 vol.-% of isopropanol and 9 vol.-% acetone were tolerated in which a significant decrease at a concentration of ≥19 vol.-% for isopropanol was observed. These findings are in line with those obtained for the effects of the released alcohols on FDH-activity (*vide supra*). In our experiments FDH-mediated deprotection succeeded on the milligram-scale when acetone was added to the reaction mixture in order to have the reaction proceed with catalytic instead of stoichiometric amounts of NADH.

Consequently this reaction appears interesting wherever formate acts as a protective group. In the following we wish to report two examples of where the transfer of the abovementioned results in FDH-mediated deformylation brought the breakthrough.

1. Release of formate protected alcohol moieties in functional organosiloxanes. The deprotection of formate protected 1,3-bis(3-formoxypropyl)tetramethyldisiloxane **7** constitutes a major challenge since the deformylation is impeded by low substrate concentrations, phase interfacial phenomena, poor substrate

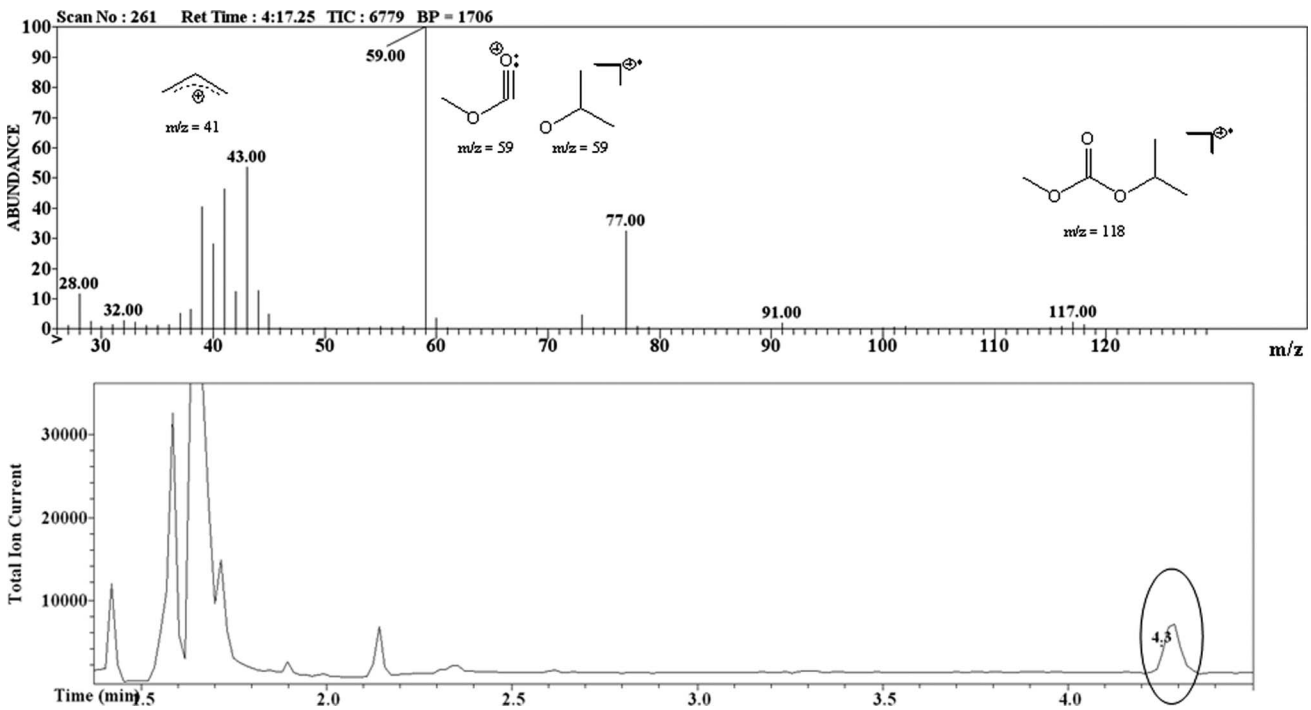
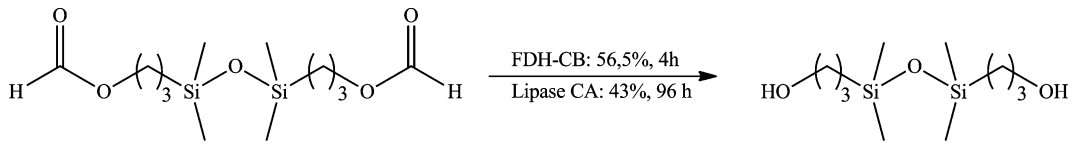


Fig. 9 GC-MS of reaction mixture from FDH-catalysed cleavage of **1**. The chromatogram above shows a peak at 4,3 min which turned out to be from methyl isopropyl carbonate as depicted in the mass spectrum below.



7 8

Fig. 11 FDH-catalysed deprotection of **7** to **8** proceeds over 56% within 4 h. In contrast, conversion does not exceed 50% after 96 h with a conventional hydrolase.

solubilities as well as low functionalisation densities. In conventional hydrolysis, lipase from *Candida antarctica* has provided the best results so far. Anyway, conversion did not exceed 43% within 96 h.[37] In contrast conversion reached 56% after only 4 h using FDH from *Candida boidinii*, and the reaction yielded the quantitatively deformylated siloxane **8** after 17 h (see Fig. 11).

Again, as pointed out above, potential contributions of hydrolase activity, in concert with FDH-mediated oxidation of intermediately released formic acid, were shown to not interfere with the FDH-mediated deformylation of **7**.

From the viewpoint of a technical application it has to be noted here that terminally functionalised α,ω-hydroxyalkylpolysiloxanes are produced from the respective formoxy protected entities what is unavoidable in order to prevent the polysiloxane backbone from being degraded through intramolecular attack of the terminal carbinol groups ("back-biting") (Fig. 12).

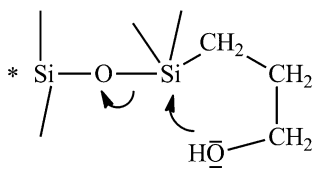


Fig. 12 Intramolecular degradation of hydroxyalkyl substituted siloxanes.

Consequently, the use of formylated hydroxyalkyl entities in concert with FDH-mediated deformylation is an economic way thus far for the production of terminally hydroxyalkyl functionalised polysiloxanes.

2. 1-Acetoxy-butane-4-ol. Another application arises from the property of FDH to excellently differentiate between C_1-carbonic acids (formic acid) and higher homologues. Since hydrolases release whatever carbonic acid from the protected alcohol, mixed esters of the type m-acyloxy-n-formoxy-alkane can easily be converted into the respective acyloxy carbinol by FDH-mediated release of CO_2.

This feature shall be exemplified with 1-acetoxy-butane-4-ol. This monoester-monocarbinol species is easily obtained by deformylation of its disubstituted progenitor **6** in single step with >99,5% conversion and yield by FDH (Fig. 4). The option to selectively modify a distinct carbinol moiety is of particular interest wherever there is no stereochemical distinction between two chemically equivalent alcohol groups (what would allow application of the so-called '*meso*-trick').[38] Both a hydrolase and a chemical catalyst like acid or base are incapable of differentiating between these two ester entities and will entirely hydrolyse those. That implies the need for the FDH to differentiate them and left the higher carbonic acid ester unreacted.

Experimental

General

Formic acid esters, FDH from *Candida boidinii* and protease inhibitor cocktail were purchased from Sigma Aldrich (St. Louis, MO, USA). Lipase Novozym 388 and NAD^+ were kindly provided by Julich Chiral Solutions GmbH–a Codexis company. All other reagents and solvents were in reagent grade and used as received without further purification.

Instruments

All reactions were monitored by gas chromatography (GC) and ^{1}H-NMR. Quantitative GC analyses were conducted using a Clarus 500 gas chromatograph from PerkinElmer Inc. (Waltham, MA, USA). Mechanistic studies were performed using an IT 400 GC-MS-system (ion trap) equipped with a FactorFour column coupled to a MS 210 ion trap mass analyser, all from Varian, Inc. (Palo Alto, CA, USA). Optical measurements were conducted on a V630 Bio UV/Vis spectrometer from Jasco GmbH (Groß-Umstadt, Germany). Reaction vessels were handled in a thermomixer (Eppendorf AG, Germany).

Synthesis of 1-acetoxy-4-formoxy butane (6)

In a two necked flask equipped with a dropping funnel 0,75 mol 1,4-butanediol were mixed with 0,50 mol pyridine at 0 °C and 0,5 mol acetic anhydride were added dropwise in order to keep temperature constant. After stirring the solution for 12 h at room temperature brine was added. After phase separation the organic layer was washed neutral and dried over sodium sulfate. After distillation at 11 mbar a clear solution with a boiling range of 76…84 °C was isolated. After removal of impurities by column chromatography on silica gel (Merck AG) with chloroform/methanol (95/5) as eluent 47,6 g 1-acetoxy-butane-4-ol were isolated (0,36 mol, 48%). ^{1}H-NMR(500 MHz, $CDCl_3$): δ (ppm) 1,57 (m, 2H), 1,67 (m, 2H), 2,0 (s, 3H), 2,45 (s, H), 3,60 (t, 2H), 4,04 (t, 2H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 171,35; 64,34; 62,11; 29,02; 25,05; 20,95. n_D^{20} = 1,4352.

In the formylation step 27 mmol copper(II) nitrate trihydrate was suspended in 18 mmol 1-acetoxy butane-4-ol and 36 mL formic acid ethyl ester and heated under reflux for 3,5 h. The solution was filtered to remove the catalyst and saturated with brine. After separation the aqueous phase was extracted three times with chloroform. The combined organic phases were dried over sodium sulfate and volatile components were removed *in vacuo*. The isolated yield was 71% in relation to 1-acetoxy butane-4-ol with a purity >97% (GC). ^{1}H-NMR(500 MHz, $CDCl_3$): δ (ppm) 1,71 (m, 2H), 1,74 (m, 2H), 2,02 (s, 3H), 4,06 (t, 2H), 4,17

(t, 2H), 8,03 (s, H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) 170,7, 160,8, 63,5, 63,1, 24,9, 20,6. MS (EI, 70 eV: m/z (%) = 161 (MH^+, 6%), 115 (58%), 101 (100%), 71 (30%), 54 (48%). Boiling point: 115 °C (10 Torr). n_D^{20} = 1,4357.

Extent of conversion

Enzymatic conversion of formic acid esters **1–6** was measured by GC on a Carbowax (FS-CW) column (50 m × 0.32 mm × 0.5 µm) from CS Chromatographie Service GmbH (Langerwehe, Germany). The pressure of the carrier gas H_2 was 0.8 bar, temperatures of injector and detector were 250 °C and 260 °C, respectively. The GC oven temperature was maintained at 50 °C for 2 min, programmed to 90 °C at 4,5 K min^{-1}, then to 180 °C at 40 K min^{-1} and maintained at 180 °C for 2 min. Standard calibration curves for all formic acid esters were plotted within a range of 1–500 mM. Mechanistic analyses were done by GC-MS measurements on a GC-431 with mass spectrometer MS-210 (ion trap, Varian Inc.) equipped with a FactorFour VF-WAXms capillary column (30 m × 0.25 mm × 0.25 µm) from Varian, Inc. (Palo Alto, CA, USA). Helium was used as carrier gas with a flow of 1 mL/min. The GC oven temperature was maintained at 50 °C for 5 min, programmed to 110 °C at 10 K min^{-1}, then to 200 °C at 40 K min^{-1} and maintained at 200 °C for 3 min.

Standard activity assay for FDH

For activity assays a standard reaction mixture was prepared by mixing 100 mM potassium phosphate buffer at pH 7.5 (1,0 mL) with 1 M sodium formate in distilled water (250 µL) and 10 mM NAD^+ in 100 mM potassium phosphate buffer (250 µL). The enzyme solution was prepared by diluting the commercial FDH stock solution in a ratio of 1 : 10 with 100 mM potassium phosphate buffer at pH 7.5. The standard reaction mixture was then kept at 30 °C in a thermomixer for 10 min. Then 50 µL of the cold enzyme solution (5 U/mL) were added to give a total reaction volume of 1550 µL. Enzymatic activity was determined after 1 min reaction time by photometric measurements of absorption at 340 nm. Linearisation and calculation of the Michaelis–Menten-constant K_M and the maximum reaction rate v_{max} were performed according to the method of Hanes.[39]

Influence of temperature and alcohol content on FDH activity

The influence of temperature was determined according to the activity assay as described ranging from 10 °C . . . 60 °C over a period of 1 . . . 5 h. Tolerance towards the cosolvent isopropanol and the alcohols released from **1–6** through the action of FDH was determined by replacing phosphate buffer by these compounds within the range of 0 . . . 50% (v/v) in accordance with the activity assay described above.

Activity of FDH towards formic acid esters

The activity of FDH at different concentrations of formic acid esters **1–6** in the range of 0,1–500 mM was analysed spectrophotometrically at 340 nm for 1–10 min. For these purposes formate ester was dissolved in isopropanol up to a concentration of 500 mM. 250 µL of formate ester were mixed with 940 µL sodium phosphate buffer (pH 7.5) and 250 µL of a 10 mM NAD^+ solution. To raise the solubility of formate esters 60 µL of isopropanol were added before the reaction was started by adding 50 µL of diluted FDH. The reaction rate and K_M-values were determined by Michaelis–Menten kinetic.

Exclusion of hydrolase-mediated side reactions

In order to ensure that the observed cleavage of esters **1–6** was not the outcome of hydrolase impurities acting on ester bonds, a differentiation assay with *p*-nitrophenyl acetate[40] was performed. Any hydrolase present in the enzyme preparation would result in a conversion of the substrate to *p*-nitrophenol which can be detected by photometric absorption measurement at 410 nm. The reaction mixture was prepared by mixing 50 mM sodium phosphate buffer (800 µL) at pH 7.5 and 10 mM *p*-nitrophenyl acetate in isopropanol (100 µL). 100 µL of the diluted enzyme solution (see above) were added and the amount of *p*-nitrophenol released was determined by immediate photometric measurement at 410 nm. The assay was performed at 30 °C. The differentiation assay was performed with and without addition of a protease inhibitor cocktail, whereas in both cases blanks were determined in an analogous manner with buffer instead of enzyme solution.

Quantitative measurement of formic acid ester cleavage

For each formic acid ester **1–6**, a 500 mM stock solution in isopropanol was prepared. The reaction mixture for quantitative measurement of formic acid ester cleavage contained 940 µL of 100 mM potassium phosphate buffer at pH 7.5, 60 µL of isopropanol, 250 µL of 10 mM NAD^+-solution (see above) and 250 µL formic acid ester stock solution in order to provide substrate and cofactor in equimolar amounts. The mixture was agitated for 10 min in a thermo mixer at 30 °C and 1000 rpm. The reaction was initiated by addition of the diluted enzyme solution (see above). In order to determine extent of conversion 200 µL samples were taken which were added 20 µL of n-hexanol (500 mM) as internal standard and diluted up to 1 mL with isopropanol. Degree of conversion was immediately determined by gas chromatography. For regeneration of NAD^+ 50 µL (5 U/mL) alcohol dehydrogenase from *Candida boidinii* (ADH-CB) and 250 µL of 550 mM acetone were added to the reaction mixture.

Conclusion

Does FDH catalyse hydrolytic reactions? Sure, but not in a conventional way. Beyond doubt the enzyme acts oxidising, although the reaction pathway bears analogies with the $A_{AC}1$ hydrolysis mechanism. Providing a final answer to mechanistic questions will certainly require further mechanistic studies, but water is definitely involved. In any case, the role of formate dehydrogenase in biocatalysis has to be reassessed. The enzyme is not only an invaluable tool in cofactor regeneration, but its catalytic activity serves well to deprotect alcohol groups esterified with formic acid. Thereby the reaction proceeds quantitatively, irreversibly and under mild conditions. With all experiments, no detrimental effects of the reaction products, which are the respective alcohols, were observed.

In view of the considerable preparative value of the FDH-method in protective group chemistry, immobilisation experiments will serve to assign our present findings to a broader application.

As can be seen from the results of conversion measurement, substrate structures determine the kinetic properties of FDH-mediated formic acid ester cleavage.

Acknowledgements

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References

1 A. Egorov, T. Avilova, M. Dikov, V. Popov, Y. Rodionov and I. Berezin, *Eur. J. Biochem.*, 1979, **99**, 569–576.
2 V. Lamzin, A. Aleshin, B. Strokopytov, M. Yukhnevich, V. Popov, E. Harutyunyan and K. Wilson, *Eur. J. Biochem.*, 1992, **206**, 441–452.
3 V. Tishkov and V. Popov, *Biomol. Eng.*, 2006, **23**, 89–110.
4 A. Bommarius, M. Schwarm, K. Stingl, M. Kottenhahn, K. Huthmacher and K. Drauz, *Tetrahedron: Asymmetry*, 1995, **6**, 2851–2888.
5 W. Hummel and M.-R. Kula, *Eur. J. Biochem.*, 1989, **184**, 1–13.
6 C. Simons, J. van Leeuwen, R. Stemmer, I. Arends, T. Maschmeyer, R. A. Sheldon and U. Hanefeld, *J. Mol. Catal. B: Enzym.*, 2008, **54**, 67–71.
7 P. Savary, *Biochim. Biophys. Acta, Lipids Lipid Metab.*, 1972, **270**, 463–471.
8 C. Reese and J. Stewart, *Tetrahedron Lett.*, 1968, **9**, 4273–4276.
9 L. De Luca, G. Giacomelli and A. Porcheddu, *J. Org. Chem.*, 2002, **15**, 5153–5155.
10 S. Koeller and J.-P. Lellouche, *Tetrahedron Lett.*, 1999, **40**, 7043–7046.
11 M. Dakanali, G. Tsikalas, H. Krautscheid and H. Katerinopoulos, *Tetrahedron Lett.*, 2008, **49**, 1648–1651.
12 H. Hagiwara, K. Morohashi, H. Sakai, T. Suzuki and M. Ando, *Tetrahedron*, 1998, **54**, 5845–5852.
13 Y. Wu and K. Houk, *J. Am. Chem. Soc.*, 1987, **109**, 2226–2227.
14 Y.-D. Wu and K. Houk, *J. Am. Chem. Soc.*, 1987, **109**, 906–908.
15 B. Schiott, Y.-J. Zheng and T. Bruice, *J. Am. Chem. Soc.*, 1998, **120**, 7192–7200.
16 V. Popov and V. Lamzin, *Biochem. J.*, 1994, **301**, 625–643.
17 A. Egorov, V. Tishkov, T. Avilova and V. Popov, *Biochem. Biophys. Res. Commun.*, 1982, **104**, 1–5.
18 L. Uotila and M. Koivusalo, *Arch. Biochem. Biophys.*, 1979, **196**, 33–45.
19 J. van Dijken, G. Oostra-Demkes, R. Otto and W. Harder, *Arch. Microbiol.*, 1976, **111**, 77–83.
20 I. Neben, H. Sahm and M.-R. Kula, *Biochim. Biophys. Acta - Enzymol.*, 1980, **614**, 81–91.
21 N. Kato, C. Sakazawa, T. Nishizawa, Y. Tani and H. Yamada, *Biochim. Biophys. Acta, Enzymol.*, 1980, **611**, 323–332.
22 T. Matsuda, R. Yamanaka and K. Nakamura, *Tetrahedron: Asymmetry*, 2009, **20**, 513–557.
23 R. Castillo, M. Oliva, S. Marti and V. Moliner, *J. Phys. Chem. B*, 2008, **112**, 10012–10022.
24 V. S. Lamzin, Z. Dauter, V. Popov, E. Harutyunyan and K. Wilson, *J. Mol. Biol.*, 1994, **236**, 759–785.
25 H. Slusarczyk, S. Felber, M.-R. Kula and M. Pohl, *Eur. J. Biochem.*, 2000, **267**, 1280–1289.
26 M. Ansorge-Schumacher, H. Slusarczyk, J. Schümers and D. Hirtz, *FEBS J.*, 2006, **273**, 3938–3945.
27 N. Kato, H. Sahm and F. Wagner, *Biochim. Biophys. Acta - Enzymol.*, 1979, **566**, 12–20.
28 *EP Pat.*, 5998, 1979.
29 S. Alivisatos, F. Ungar and G. Abraham, *Nature*, 1964, **203**, 973–975.
30 S. Alunni, A. Cipiciani, G. Fioroni and L. Ottavi, *Arch. Biochem. Biophys.*, 2003, **412**, 170–175.
31 M. Stockdale and M. Selwyn, *Eur. J. Biochem.*, 1971, **21**, 416–423.
32 A. Serov, A. Popov and V. Tishkov, *Dokl. Biochem. Biophys.*, 2002, **382**, 26–405.
33 C. Ingold, *in Structure and Mechanism in Organic Chemistry*Cornell University Press, Ithaca, New York, 1969.
34 J. Berg, J. Tymoczko, L. Stryer, *in Biochemistry*W. H. Freeman & Co Ltd, New York, 2006.
35 J. Peters, T. Zelinski, T. Minuth and M.-R. Kula, *Tetrahedron: Asymmetry*, 1993, **4**, 1683–1692.
36 T. Stillger, M. Bönitz, M. Villela Filho and A. Liese, *Chem. Ing. Tech.*, 2002, **74**, 1035–1039.
37 P. Fröhlich and M. Bertau, *Chem. Ing. Tech.*, 2010, **82**, 51–63.
38 G. Gamalevich, B. Morozov, A. Vlasyuk and E. Serebryakov, *Tetrahedron*, 1999, **55**, 3665–3674.
39 C. Hanes, *Biochem. J.*, 1932, **26**, 1406–1421.
40 V. Khalameyzer, I. Fischer, U. Bornscheuer and J. Altenbuchner, *J. Appl. Environ. Microbiol.*, 1999, **65**, 477–482.

