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Pyrococcus furiosus-mediated reduction of conjugated carboxylic acids: Towards using syngas as reductant

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ABSTRACT

Pyrococcus furiosus catalyzes the reduction of carboxylic acids to their corresponding alcohols. In addition to hydrogen also carbon monoxide can be used as stoichiometric reductant, paving the way to cheap syngas to promote biocatalytic acid reduction. The enzymes responsible for coupling CO-oxidation to acid reduction are currently unknown but may represent an unprecedented enzyme class. Furthermore, enoate reductase-like activity has been detected in *P. furiosus* while lacking 'classical' enoate reductases.

1. Introduction

The reduction of carboxylic acids to aldehydes or alcohols represents an important functional group transformation in organic chemistry. On the laboratory scale, metal hydrides prevail as stoichiometric reductants of choice [1]. Evidently, from an atom-efficiency-point-of-view hydrogen gas represents an ideal reducing agent. In fact on industrial scale, catalytic hydrogenation is well established, e.g. for the production of fatty alcohols. However, vigorous reaction conditions are typically required due to the low reactivity of the carboxylic group. Also, undesired side reactions, such as the over-reduction to hydrocarbons and esterification of the product with the starting material, are notoriously hard to suppress in the course of transition-metal based hydrogenations.

Biocatalytic hydrogenation may be a viable alternative to the above-mentioned methods due to the generally higher selectivity of enzymes and the milder reaction conditions. However, compared to the reduction of, e.g. carbonyl groups [2] or conjugated C=C-double bonds [3], the biocatalytic reduction of carboxylates is fairly underrepresented [2c]. Some decades ago Simon and coworkers pioneered microbial hydrogenation reactions (including some carboxylic acids) [4]. Unfortunately, the preparative impact of these studies has been very limited. More recently Rosazza and coworkers established NADPH- and ATP-dependent reduction of carboxylic acids using 'so-called' acid oxidoreductases [5]. Very recently, we demonstrated that *Pyrococcus furiosus* is able to reduce a range of carboxylic acids using H_2 as stoichiometric reductant [6]. Here, hydrogenase (Hase)-mediated oxidation of H_2 [7] was productively coupled to an aldehyde oxidoreducase (AOR)-mediated reduction of carboxylic acids [8] and further reduction to the corresponding alcohols as catalyzed by NAD(P)H-dependent alcohol dehydrogenases (Scheme 1). AOR family enzymes have been established to have carboxylic acid reduction activity in vitro [4b,4c,9] *P. furiosus* has five AORs on the genome, which have all been biochemically characterized [10].

However, if the *P furiosus*-mediated reduction of carboxylic acid would be strictly dependent on pure hydrogen gas, its synthetic usefulness would be severely limited by the high price of pure H_2. In fact, synthesis gas (being a mixture of H_2 and CO at various ratios) would represent a very cheap and readily available reducing agent. On the one hand a major concern arises from the high toxicity of CO for many living systems. On the other hand, 'so-called' CO-dehydrogenases (CODHs) have been reported, e.g. in *Clostridium thermoaceticum* [4d,11]. Also, very recently Angenent and coworkers reported the use of syngas as reductant in *Clostridium*-promoted reduction of short-chain carboxylic acids [12].

The aim of the experiments reported in this communication therefore was to investigate whether *P. furiosus* could tolerate or maybe even utilize CO to reduce carboxylic acids (Scheme 1).

2. Materials and methods

2.1. Chemicals

All carboxylic acids, aldehydes, ketones and alcohols were obtained from Sigma–Aldrich or Alfa Aesar in the highest purity available and used without further purification.

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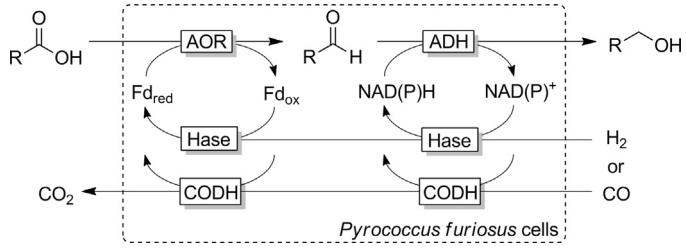


Scheme 1. Suggested *P. furiosus*-mediated reduction of carboxylic acids with H_2 or CO as reductant. AOR: aldehyde oxidoreductase; ADH: alcohol dehydrogenase; Hase: hydrogenase; CODH: CO dehydrogenase; Fd_{red}/Fd_{ox}: reduced/oxidized ferredoxin.

2.1.1. Preparation of *P. furiosus* cells

P. furiosus (DSM 3638) was grown in a 100 L fermenter at 90 °C, under anaerobic conditions with potato starch as carbon source as previously described [6]. Cells were harvested by crossflow filtration and centrifugation and stored at −80 °C for further use.

2.2. General reaction conditions

For our investigations we utilized the reaction conditions previously identified to be suitable for the *P. furiosus*-catalyzed hydrogenation of carboxylic acids [6]. In short: reaction mixtures of 2 ml in 16 ml autoclaves containing 0.3 g *P. furiosus* frozen cells, 10 mM carboxylic acid substrate and 100 mM sodium phosphate buffer (pH 6.5) were flushed with N_2 and pre-purged with H_2 (p = 5 bar). A photograph of the experimental setup is shown in the supporting information. The reactions were incubated at 40 °C with magnetic agitation at 100 rpm for 24 h. The reaction mixture was acidified to pH 2.0 with 5 N HCl, extracted twice with distilled ethyl acetate or diethyl ether containing 1-octanol or *n*-decane as an internal standard, and analyzed by GC. In the case of some aromatic acids, the samples were centrifuged for 15 min at 13,000 rpm after adding equal volumes of acetonitrile, and the supernatant was analyzed by HPLC.

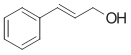
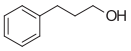
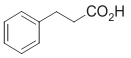
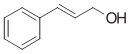
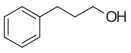
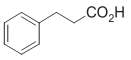
2.3. Analytical procedures

The reaction progress as well as the optical purity of the products was determined using GC analysis or HPLC. GC was performed using a CP-Sil 5 CB column (50 m × 0.53 mm × 1.0 μm) or a CP-Wax 52 CB (50 m × 0.53 mm × 2.0 μm) with N_2 as carrier gas and flame ionization detector. HPLC analysis was carried out with a Waters Xterra column (C18, 5 μm, 4.6 × 150 mm) with CH_3CN:H_2O:HCOOH (20:80:1, v/v/v) as eluent. The samples were isocratically eluted at a flow rate of 1 ml/min.

2.4. BLAST searches

The genome of *P. furiosus* [13] was searched for the following sequences to identify putative (1) enoate reductases or (2) CO-dehydrogenases. To scan for enoate reductases three representatives of the two subfamilies of the Old Yellow Enzyme (OYE) family and two representatives of the *Clostridial* enoate reductases were used: *Saccharomyces pastorianus* OYE1 as a representatives of the classical OYE, and *Bacillus subtilis* YqjM and *Thermus scotoductus* SA-01 Chromate reductase as representatives of the thermophilic-like OYE, and *Clostridium tyrobutyricum* and *Clostrium ljungdahlii* as representatives of the *Clostridial* enoate reductases [3a,14] Furthermore, in order to scan for putative CO-dehydrogenases two different CO-dehydrogenase representatives that have been structurally and biochemically characterized have been used: *Oligotropha carboxidovorans* Mo-dependent CO-dehydrogenase and *Carboxydothermus hydrogenoformans* Ni-dependent CO-dehydrogenase [15]. Details can be found in the supporting information.

Table 1
Comparison between H_2 and CO as reductants for the *P. furiosus*-catalyzed reduction of cinnamic acid.[a]

Product	Yield [%]		
	pH 5.5	pH 6.5	pH 8.0
H_2 as reductant			
	0	40.3 ± 1.8	2.7 ± 0.2
	0	29.1 ± 1.3	10.4 ± 0.7
	3.7 ± 0.1	8.2 ± 0.8	67.7 ± 5.9
CO as reductant			
	0	37.2 ± 1.6	0.7 ± 0.1
	0	16.1 ± 1.0	1.7 ± 0.1
	0.6 ± 0	8.3 ± 1.3	78.3 ± 3.5

Reaction conditions were: 100 mM sodium phosphate buffer (pH 6.5), T = 40 °C, c(substrate) = 10 mM, p(H_2 or CO, respectively) = 5 bar, c(*P. furiosus*) = 0.15 g mL^{-1}.

3. Results and discussion

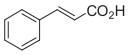
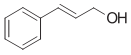
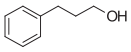
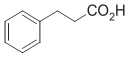
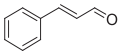
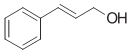
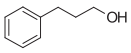
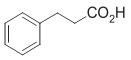
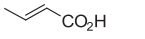
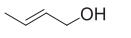
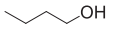
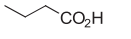
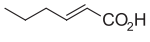
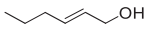
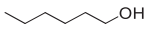
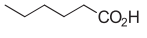
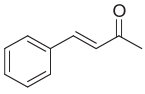
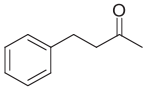
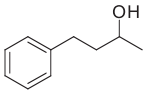
To approach the question whether *P. furiosus* might utilize CO as a sacrificial electron donor we used cinammic acid as a model substrate and compared the product distribution using H_2 and CO as reductants under otherwise identical reaction conditions (Table 1).

We found that *P. furiosus* not only tolerated CO but also utilized it as sacrificial electron donor. As shown in Table 1 conversion and product distributions were essentially identical using either H_2 or CO as reductant. It is worth mentioning here that no significant conversion was observed in the absence of either CO or H_2 (but under 5 bars of N_2). Currently, we can only speculate about the electron transport chain enabling *P. furiosus* to utilize CO as reductant. Possibly a CO dehydrogenase (CODH) as suggested for *C. thermoaceticum* [4d,11] might also be present in *P. furiosus*. However, no indications for homologues of the known Mo- or Ni-dependent CODHs (PDB: 1ZXI_E and 1SU6_A) were found in the genome of *P. furiosus* [13]. It should be noted that the Ni-CODH has sequence homology to parts of the coding sequence of the Hybrid cluster protein (HCP), which exists in *P. furiosus* [16]. The only established enzymatic activity of HCP is hydroxylamine reduction to ammonia. Further work in our laboratory will concentrate on isolating and characterizing the putative CODH of *P. furiosus*. In any case, these findings point towards the usage of much cheaper synthesis gas instead of highly purified H_2 as reductant. Also we like to interpret the identical results using H_2 and CO by assuming that the thermodynamically challenging reduction of carboxylic acids is overall rate-limiting, which is also in line with the finding that the intermediate aldehyde was not observed.

Another interesting finding from these experiments is the comparably poor chemoselectivity as significant amounts of the saturated carboxylic acid and alcohol were observed. In previous experiments we found that non-conjugated C=C-double bonds were not converted by *P. furiosus* [6].

Enoate reductases (ERs, E.C. 1.3.1.31) are well-known to catalyze the reduction of conjugated C=C-double bonds to the corresponding saturated carbonyl compounds (especially aldehydes) [3],

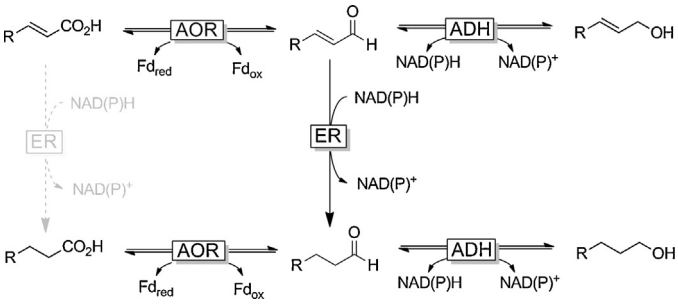
Table 2
P. furiosus-mediated hydrogenation of α,β-unsaturated carbonyl compounds.

	Substrate	Product	Yield [%]
1			40.3 ± 1.8
			29.1 ± 1.3
			8.2 ± 0.8
2			16.2 ± 1.0
			63.5 ± 1.8
			4.6 ± 1.5
3			14.9 ± 0.4
			18.2 ± 0.5
			32.5 ± 1.3
4			31.4 ± 1.5
			51.7 ± 2.2
			14.5 ± 2.2
5			80.1 ± 0.3
			Traces
6			57.1 ± 0.5
			Traces

Reaction conditions were: 100 mM sodium phosphate buffer (pH 6.5), $T = 40\,^{\circ}C$, c(substrate) = 10 mM, $p(H_2) = 5$ bar, c(*P. furiosus*) = 0.15 g mL^{-1}.

which may explain the poor chemoselectivity observed. Therefore, we also evaluated some typical ER-substrates such as cyclohexenone and *trans*-4-phenyl-3-buten-2-one. Indeed, also these test compounds were smoothly converted by *P. furiosus* (Table 2) indicating the presence of at least one endogeneous ER in *P. furiosus*. We hypothesize that one (or more) endogeneous ERs within *P. furiosus* may reduce especially the α,β-unsaturated aldehyde occurring in the reduction sequence, possibly also C=C-bond reduction of the less activated α,β-unsaturated carboxylic acid may occur.

This enzyme cascade may also explain the observation that the ratio between saturated alcohol and allylic alcohol (Table 2, entries 1 and 2) seem to vary significantly upon changing from conjugated carboxylic acid to the corresponding aldehyde as starting material. This ratio essentially is determined by the relative rate of C=O- and C=C-reduction (mediated by ADHs and ERs, respectively). Provided the apparent K_M-value for carbonyl reduction is higher than the corresponding K_M for C=C-reduction this would explain the decreased C=C-reduction activity in case of the carboxylic acid as



Scheme 2. Hypothesized enzymes involved in the reduction of α,β-unsaturated acids. Aldehyde oxidoreductases (AOR) mediate the (reversible) reduction of the carboxylate groups whereas alcohol dehydrogenases catalyze the 'through-reduction' to the corresponding alcohols. Putative enoate reductases (ER) catalyze the C=C-bond reduction of the conjugated acids and aldehydes. As conjugated carboxylic acids are generally far less reaction in C=C-bond reductions [3a] we postulate that the C=C-bond reduction proceeds primarily via the α,β-unsaturated aldehyde.

starting material (please note that the reduction of the carboxylate group is overall rate limiting resulting in a very low in situ concentration of the conjugated aldehyde).

Another interesting observation made with cinnamaldehyde as starting material is the accumulation of significant amounts of the saturated acid. This was somewhat unexpected under the reducing conditions applied in these experiments but is plausible considering the high thermodynamic driving force of the oxidation direction. Currently, the nature of the terminal electron acceptor remains elusive, but it may be hypothesized that *P. furiosus* metabolites may serve as 'sacrificial electron acceptors'. Overall, the occurrence of 3-phenyl propionic acid from cinnamaldehyde may be rationalized as a sequence of ER-catalyzed reduction of the C=C bond followed by AOR-catalyzed oxidation (Scheme 2). It is worth mentioning here that the formation of traces of saturated acid from conjugated aldehydes appears to be a rather common property of *P. furiosus* as it was also observed with other α,β-unsaturated aldehydes (see supporting information for more details).

Interestingly again, a BLAST search within the genome of *P. furiosus* [13] revealed no evidence for an enoate reductases belonging to the old yellow enzyme family. Only several homologs to a part of the *Clostridial* enoate reductases were found. These *Clostridial* enoate reductases contain two distinct domains: an N-terminal part, which constitutes 2/3 of the overall protein and is related to OYE (ER_like_FMN conserved domain cd02931) and a C-terminal 1/3, which is a Pyr_redox domain (conserved domain Pfam00070) [17]. A blast search of sequences of representative enzymes of 2-enoate reductase from *C. tyrobutyricum* and *C. ljungdahlii* against the *P. furiosus* genome resulted in several homologs to the C-terminal part of the enoate reductases only. These homologs Pf1327, Pf1532, Pf1795 and Pf1910 are all uncharacterized NAD(P)H dependent oxidoreductases (the amino acid sequences are given in the supporting information). These enzymes lack the FMN and FeS cluster binding sites of the *Clostridial* enoate reductase and, therefore, most likely have a different function. Possibly, this may indicate the existence of a novel class of ER such as NAD(P)H-dependent non-flavin-ERs [18]. Further studies on identifying the enzyme(s) responsible for ER-like activity are currently underway.

4. Conclusions

P. furiosus remains an organism with many surprises. In the present study we have demonstrated that CO can be utilized as stoichiometric reductant by *P. furiosus*, e.g. for the reduction of carboxylic acids. On the one hand, this finding may pave the way to a syngas-based reduction chemistry utilizing *P. furiosus*. On the other hand, this unexpected finding suggests the existence

of currently unknown CO-dehydrogenases in *P. furiosus*, which may exhibit some potential as robust biocatalysts to promote biocatalytic reduction reactions. The mechanism of CO oxidation and its productive coupling to, e.g. acid reduction remains to be demonstrated and investigations aiming at the isolation and characterization of the putative CODH are currently underway in our lab.

Also, enoate reductase-like activities have been detected in *P. furiosus* while the genome of this organism does not contain genes homologous to 'classical' ERs. This may indicate the presence of a novel ER possibly of practical usefulness. Isolation and characterization of these putative ERs are currently underway.

Admittedly, the system presented here is relatively premature and many optimization steps (e.g. increased substrate loadings and/or use permeabilized cells to minimize diffusion limitation, to mention a few) are necessary *en route* to a truly practical synthetic tool. Also the poor chemoselectivity observed with conjugated carboxylic acids calls for further improvements such as use of purified enzymes. Despite the fact that it 'is still a long way to go' we are convinced that *P. furiosus* (and its enzymes) exhibit are great potential to 'new' enzyme activities and chemical transformations unprecedented amongst the established enzymes.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2013.09.006.

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