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Alcohol dehydrogenases catalyze the reduction of thioesters

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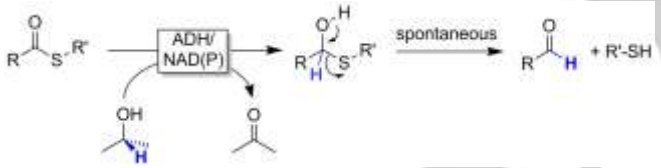
Abstract: Alcohol dehydrogenases are well-established catalysts for various reduction reactions. However, reduction of carboxylic acid derivatives has not been reported with these enzymes yet. In this contribution we demonstrate that carboxylic acid thioesters are readily reduced by a range of alcohol dehydrogenases, albeit at significantly reduced rates. A molecular explanation especially for the lower k_{cat} values towards thioesters as compared to ketones is presented and a preliminary substrate scope is presented.

The reduction of carboxylic acids remains a rather underrepresented area in catalysis research.[1] The methods of the state-of-the art often suffer from poor atom-efficiency and poor chemoselectivity.

In this respect biocatalysis may fill the gap by providing highly selective catalysts for the reduction of carboxylates. However, the biocatalytic reduction of carboxylic acids, despite being known since at least the 1950s,[2] is fairly underrepresented. Today, mostly carboxylic acid reductases (CARs)[2a, 3] and aldehyde oxidoreductases (AORs)[4] are under investigation and highly chemoselective reduction of only the carboxylic acid moiety has been demonstrated for both.

The catalytic mechanism of CARs entails the (ATP-driven) *in situ* formation of a thioester (phosphopantetheine ester), which then is reduced in an alcohol dehydrogenase-like reaction to the corresponding hemithioacetal which spontaneously rearranges into the free thiol and aldehyde. This mechanism inspired us to evaluate whether alcohol dehydrogenases might be capable of reducing thioesters (Scheme 1). This not only would expand the reaction scope of this well-known class of enzymes but also would offer new possibilities for biocatalytic acid reduction.

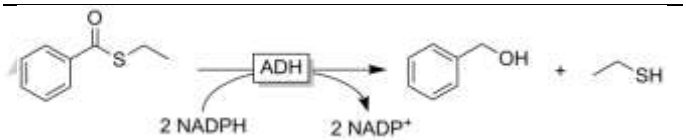


Scheme 1. ADH-catalyzed reduction of thioesters to the corresponding thio-hemiacetal which spontaneously rearranges to the aldehyde and thiol.

Our assumption was fuelled by the ^{13}C carbonyl shifts of thioesters, which are more ketone-like compared to acids and esters (Figure S1). This encouraged us to test thioesters with alcohol dehydrogenases (ADHs).

In a first set of experiments we evaluated a set of ADHs for their activity on ethyl thiobenzoate. In particular the ADHs from *Ralstonia* species (*Ras*ADH),[5] *Sphingobium yanoikuyae* (*Sy*ADH),[5c, 6] *Lactobacillus brevis* (*Lb*ADH),[7] *Lactobacillus kefir* (*Lk*ADH),[7] and *Rhodococcus ruber* (*Rr*ADH-A)[8] were recombinantly expressed in *Escherichia coli* and tested as crude cell extracts. As shown in Table 1 *Ras*ADH, *Sy*ADH and *Lb*ADH gave clear conversion of the starting material into benzyl alcohol. Crude extracts containing *Lk*ADH of *Rr*ADH-A as well as crude extracts of 'empty' *E. coli* cells showed no conversion of ethyl thiobenzoate, which was recovered quantitatively after the reaction.

Table 1. Screening various ADHs for conversion of ethyl thiobenzoate.



Enzyme	[ethyl thiobenzoate] [mM]	[benzyl alcohol] [mM]
*Ras*ADH	5.2	4.4
*Sy*ADH	9.1	0.9
*Lb*ADH	9.7	> 0.1
*Lk*ADH	10	0
*Rr*ADH-A	10	0

*Ras*ADH: *Ralstonia* sp. ADH; SyADH: *Sphingobium yanoikuyae* ADH; *Lb*ADH: *Lactobacillus brevis* ADH; *Lk*ADH: *Lactobacillus brevis* ADH; *Rr*ADH-A: *Rhodococcus ruber* ADH-A; Conditions: 1 mL potassium phosphate buffer (50 mM, pH 7.5); $[ethylthiobenzoate]_0$=10 mM, $[NAD(P)H]_0$=10 mM; 10% (v/v) isopropanol; 25% (v/v) crude extract, T=30°C, reaction time: 20h. Note that no other products than benzyl alcohol were observed via GC.

It is also interesting to note that neither benzoic acid (hydrolysis product) nor benzaldehyde (primary reduction product) was detectable. While the first observation is in line with the well-known kinetic stability of thioesters against hydrolysis the non-detectability of the intermediate benzaldehyde suggests that the first reduction step was overall rate-limiting and slower than the aldehyde reduction step. Quite expectedly, benzoic acid or its ethyl ester were not converted at all.

Encouraged by these results we further evaluated the scope of thioesters converted by *Ras*ADH (Table 2). In accordance to the published substrate scope of *Ras*ADH[5a] we focused on aromatic thioesters as substrates.

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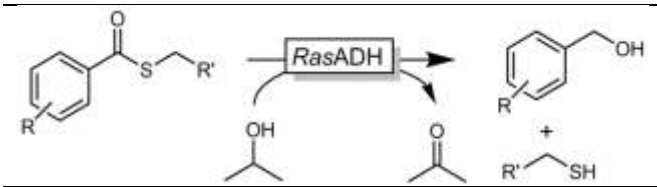
Table 2. Preliminary substrate scope of the *Ras*ADH-catalyzed reduction of thioesters.



R	R'	Conversion [%][a]
-	CH_3	84
-	CH_2CH_3	82
-	$(CH_2)_2CH_3$[b]	5.2 ± 0.1
-	$(CH_2)_3CH_3$[b]	0
p-NO_2	CH_3	8 ± 1.1[c]
p-Cl	CH_3	100 ± 1.8
p-F	CH_3	90 ± 0.1
p-OCH_3	CH_3	80 ± 1.0
p-NH_2	CH_3	0
o-Cl	CH_3	100 ± 1.8
o-F	CH_3	100 ± 1.8
o-Br	CH_3	100 ± 1.8
o-OCH_3	CH_3	42 ± 0.1
m-F	CH_3	100 ± 0.6
m-OCH_3	CH_3	64 ± 1.2
m-NH_2	CH_3	0

Conditions: [lyophilized cells] = 20 gL^{-1}, [substrate] = 10 mM, [NADPH]= 1 mM, [isopropanol]=10% v/v in 1ml phosphate buffer (pH7.5) at 30°C for 24h. [a]: only starting material of the corresponding alcohols were detected; [b]: [substrate] 1 mM due to poor solubility; [c]: significant amounts of the hemithioacetal were detected.

Increasing the chain-length of the thiol side-chain significantly reduced the conversion rate of the corresponding thioesters, which we attribute to its increasing steric demand. Apart from this, a broad range of substituted aromatic acid derivatives was converted in accordance with the substrate scope of *Ras*ADH. Scaling up the reductions of ethyl thiobenzoate and ethyl (*p*-Cl-thiobenzoate) to 10 mmol-scale was successful with isolated yields of 900 mg and 1.2 g of the corresponding benzylalcohols.

Next, we compared the rates of *Ras*ADH-catalyzed reduction of ethyl thiobenzoate, the corresponding ethyl ester and ketone (butyrophenone) (Figure 1). Expectedly, no conversion of the benzoic acid ester was observed while practically full conversion was observed for both butyrophenone and ethyl thiobenzoate. However, while the ketone was converted smoothly, reduction of the thioester was significantly slower.

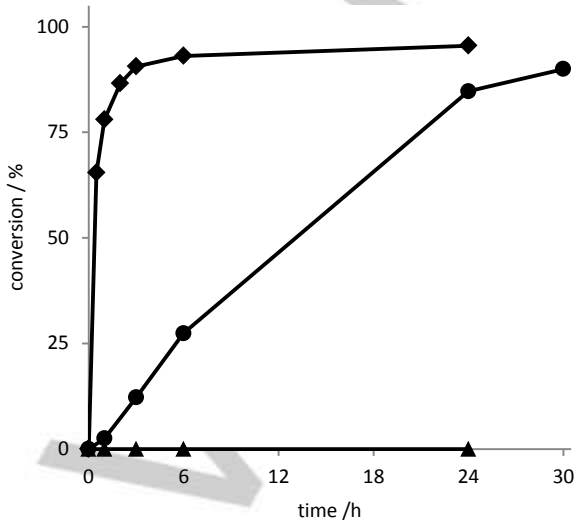


Figure 1. Time-courses of the *Ras*ADH-catalyzed reduction of butyrophenone (◆), ethyl thiobenzoate (●) and benzoic acid ethyl ester (▲) using lyophilized E. coli cells containing recombinant *Ras*ADH. Conditions: 50 mM KPi buffer (pH7.5), (10 % v/v) 2-propanol, [$NADP^+$] = 1 mM, c(*E.coli*) = 20 gL^{-1}, c(starting material)$_0$=10 mM, T=30°C.

This motivated us to determine the Michaelis-Menten Kinetics of the reduction reactions (Table 3 and Figures S2 and S3).

Table 3. Comparison of the kinetic parameters of the *Ras*ADH-catalyzed reduction of butyophenone and ethyl thiobenzoate.[a]

Substrate	**K_M [mM]**	**V_{max} [µmol min^{-1} mg^{-1}]**	**K_{cat}/K_M [s^{-1} mM^{-1}]**
Butyrophenone	1.46 ± 0.01	0.364 ± 0.013	0.312
Ethyl thiobenzoate	13.6 ± 1.5	$7.4*10^{-4}$ ± $0.3*10^{-4}$	$6.7*10^{-5}$

[a] see supporting information for detailed information.

While the affinity of *Ras*ADH towards ethyl thiobenzoate compared to the corresponding ketone was reduced 'only' 10-fold (approx. 10 times higher K_M value) the maximal conversion rate (v_{max}) was reduced almost by a factor of 500 (i.e. 9.8 *Ras*ADH turnovers per minute compared to 0.02 min^{-1}). This poor activity cannot entirely be attributed to a poorer carbonyl activity of the thioester as CARs convert comparably deactivated thioesters with specific activities in the range of 1 U mg^{-1} (corresponding to TFs of more than 120 min^{-1}).[3d]

To further understand this difference in reactivity we modelled the interaction of butyrophenone and ethyl thiobenzoate into the active site of *Ras*ADH using the crystal structure of the *Ras*ADH-NADPH complex (PDB: 4BMS).[5c] A comparison of both docking results revealed that the thioester binds to the active site in a slightly distorted orientation towards

the nicotinamide moiety of the bound NADPH (Figure 2). As a result, the distance of the NADPH-*proS*-hydride to the carbonyl group increases from 2.8 Å in case of propiophenone to 3.8 Å with ethyl thiobenzoate, also due to the distorted orientation an ideal Bürgi–Dunitz angle (64° for thiobenzoate and 92° for the ketone) cannot be obtained. Furthermore, essential H-bonds (e.g. Y150 and S137) to the carbonyl-O-atoms are prolonged significantly. This explains that suboptimal binding of the thioester substrates and unfavourable orientation of the thioester carbonyl group relative to the nicotinamide moiety mainly accounts for the poor activity observed.

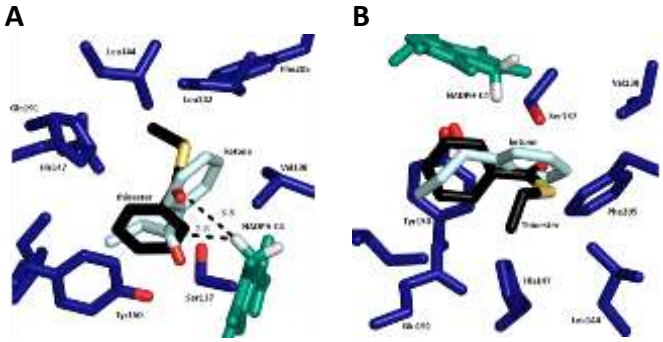


Figure 2. A) Overlay of the thioester substrate and the natural ketone substrate modelled into the active site of *Ras*ADH. B) Repulsion of the thioester substrate caused by the bulky residue Phe205. Propiophenone is shown in light blue and the thiobenzoate is coloured in black with the sulphur atom in gold and the oxygen atom from the carbonyl group in red. The important active site residues are shown in dark-blue and the NADPH in green. Important hydrogens are shown in white. The distances between the C4H of the NADPH and the carbonyl-C-atoms of the substrates are given in black dashed lines with the respective distances

Particularly, Phe205 appears to be responsible for this distorted binding especially of the sterically demanding thioester substrates. Therefore, we believe that especially iterative site mutagenesis methods[9] may result in more active enzyme variants.

Overall, in the current contribution we have demonstrated that thioester derivatives of carboxylic acids are transformed by alcohol dehydrogenases for NAD(P)H-dependent reduction thereby enlarging the reaction scope of this well-known enzyme class. The thioesters evaluated in this study have been synthesized using a modification of a published procedure[10] from the corresponding carboxylic acids and thiols in a one-step procedure and could be applied to the enzymatic reduction without further purification. Nevertheless, a more atom-efficient procedure is highly desirable. Therefore, next to protein engineering to get more active variants, also an integrated reaction system combining *in situ* thioesterification [11] and hence use of the thiol in catalytic amounts will be necessary to attain preparative relevance.

Experimental Section

Experimental details can be found in the electronic supporting information.

Acknowledgements

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Keywords: Biocatalysis • Thioester • Reduction • Alcohol dehydrogenases • Acid

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