Enzyme stabilizer DTT catalyzes nitrilase analogue hydrolysis of nitriles

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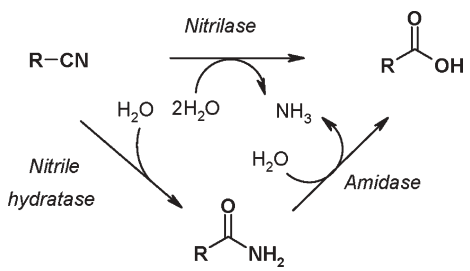
Received (in Cambridge, UK) 29th November 2005, Accepted 23rd January 2006
First published as an Advance Article on the web 10th February 2006
DOI: 10.1039/b516937b

Amides are the dominating products in some nitrilase catalyzed conversions of α-activated nitriles, but unexpectedly this hydrolytic reaction is also catalyzed by 1,4-dithio-DL-threitol (DTT), a standard antioxidizing enzyme stabilizer.

Enzymatic nitrile hydrolysis offers a mild alternative to the harsh chemical hydrolysis conditions. Two different types of enzymes (*i.e.* nitrilases and nitrile hydratases) catalyze efficient nitrile hydrolysis to carboxylic acids and/or carboxylic acid amides chemo-, regio-, diastereo- and enantioselectively[1] (Scheme 1).

We have recently prepared β-amino acids/amides by nitrile hydratase catalyzed biotransformation[2] and became interested in nitrilase mediated transformations of these compounds. Nitrilases belong to the enzyme class of hydrolases (EC 3.5.5.1) and typically directly release the carboxylic acid in contrast to the two step conversion of the nitrile hydratase/amidase enzyme system. A well accepted enzymatic mechanism describes the sulfhydryl group of a cysteine residue in the active site to bind to the nitrile. A tetrahedral intermediate is formed and usually this intermediate breaks into the corresponding carboxylic acid under the release of ammonia.[3] However, few reports address also the formation of amides,[4] which appears to result from an unusual breakdown of the tetrahedral intermediate (Scheme 2).

As no crystal structure of any biocatalytically active nitrilase has been published to date, only speculations about the mechanism of this breakdown exist. It is our aim to investigate the structural prerequisites which favor this amide-formation. A few literature reports gave rise to the assumption that substrates with electron withdrawing groups in the α-position to the nitrile are prone to give amides. A series of α-fluoro- and α,β-unsaturated nitriles were converted to their amides by an *Arabidopsis thaliana* nitrilase,[5] but also β-cyano-L-alanine was reported to give about 60% of the corresponding amide with a nitrilase of *Arabidopsis thaliana*.[6]



Scheme 1

We prepared α-activated nitriles **1a** (a precursor of the Taxol side chain[7]) and **2a** (Fig. 1), and subjected them to eight commercially available nitrilases NIT-101–NIT-108 (Biocatalytics Inc., CA). The experiments were carried out according to their proposed procedure: 1 mg of enzyme preparation was dissolved in 497.5 μL of the assay buffer [50 mM phosphate, pH 8.0, 1 mM Na_2EDTA as protease inhibitor and 2 mM 1,4-dithio-DL-threitol (DTT) as antioxidant in deionized water]. To this enzyme solution 2.5 μL of a solution of the nitrile in DMSO was added to give a final concentration of 0.2 mM. The reactions were stopped by adding ethyl acetate and the products isolated by extraction. Conversions were analyzed by HPLC.[8]

As expected, preliminary experiments proved the formation of amides from substrates **1a** and **2a**, see Table 1. Nitrile **1a** is poorly accepted as substrate by NIT-101–NIT-103 but excellently by NIT-104–NIT-108. Nitrile **2a** is only accepted by NIT-106.

To our surprise, significant amounts of the respective amides were detected in control experiments mixing only the substrate in cosolvent with phosphate buffer, EDTA and DTT. Finally, DTT turned out to be responsible for the amide formation in the blank experiments (Table 1, entries 6–8). Experimentally this result was confirmed by incubating the substrates for 18 h in the screening buffer with different DTT concentrations added, see Fig. 2. DTT can possibly also act in a mechanism comparable to the nitrilase mechanism depicted in Scheme 2.[9] Thus, it was likely that other α-activated substances such as the typical nitrilase substrate mandelonitrile **3a** (Fig. 1) are also converted to the amide by DTT. The respective experiments evidence the formation of mandelic amide in significant amounts (Fig. 3), even on a 100-fold molar scale but the same DTT/substrate ratio[10] compared to **1a** and **2a**.

Subsequent experiments with enzymes were thus performed in the same buffer but the additive DTT was omitted. Nevertheless, the respective amides were still found as major products.

Since DTT is frequently used for enzyme stabilization[11] (especially nitrilases are susceptible to thiol oxidation in the active site), we purified the enzymes from any low molecular weight substances by repeated ultrafiltration. Not surprisingly there was a comparable amount of amide formed by nitrilases where DTT or similar small size compounds were removed *versus* the standard experiment (Table 1, entry 2 and 3, entry 4 and 5, entry 9 and 10).

All our experiments evidence that not only DTT is responsible for amide production but also the nitrilases themselves catalyze

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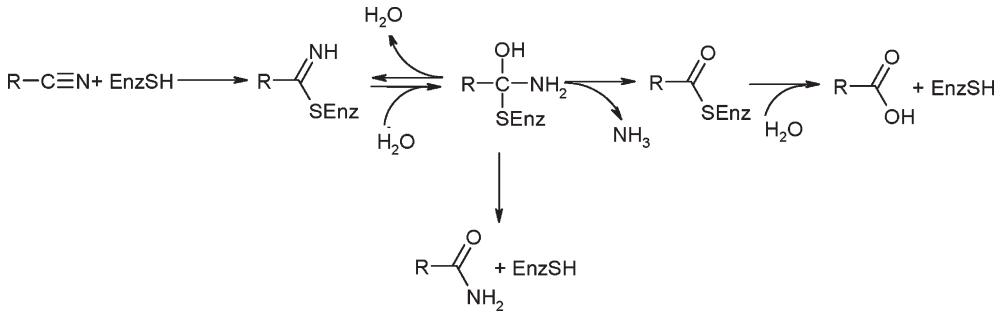

Scheme 2 Proposed mechanism of nitrilase mediated nitrile hydrolysis.

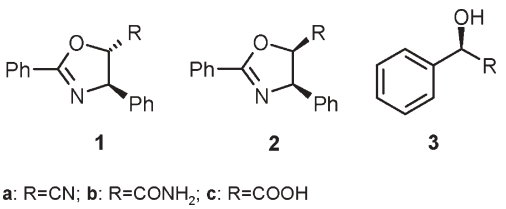

Fig. 1 Racemic substrates for amide formation (only one enantiomer is depicted).

this non-conventional reaction to amides instead of carboxylic acids. If DTT is present in addition to nitrilases, the yields of amide can be elevated but this is not generally the case, since higher amounts of DTT also decrease the enzyme activity.[6] Low concentrations of DTT, as usually included in the enzyme preparations for long-time storage, showed moderate influence in our experiments.

In summary, we found that the enzyme stabilizing agent DTT catalyzed non-enzymatic amide formation from α-activated nitriles. Apart from enzymatic reactions the ease of this amide formation in aqueous buffer at neutral pH renders this synthesis a mild alternative to the classical nitrile hydrolysis to primary amides. Considering that DTT is a chiral compound, asymmetric routes to enantioenriched amides also seem to be possible.

An activation at the α-carbon appears to be a stuctural requirement, which is supported by the fact that α-unactivated β-amino nitriles[2] are neither converted to amides by nitrilases nor by DTT.[12]

We gratefully acknowledge the Austrian Science Fund (project No. P-15810) for financial support.

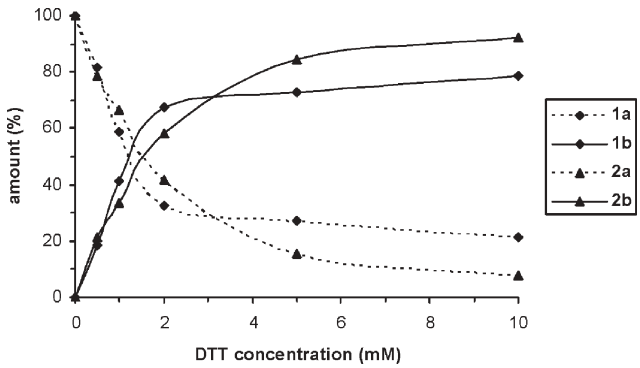

Fig. 2 Effect of the DTT concentration on conversion of α-activated nitriles **1a** and **2a** after 18 h in 50 mM phosphate buffer (pH 8.00, 1 mM EDTA) without enzyme.

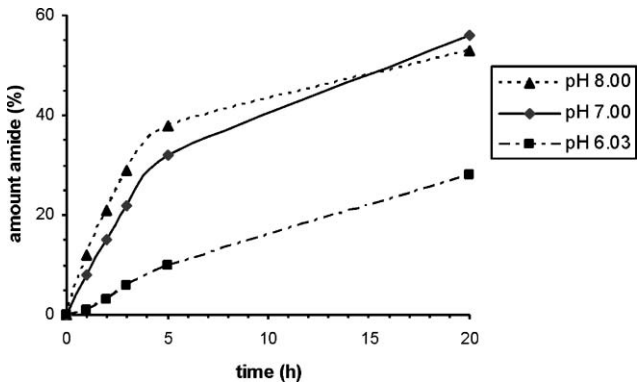

Fig. 3 Non enzymatic, DTT mediated conversion of mandelonitrile **3a** to mandelic amide **3b** in 50 mM buffer at different pH values.

Table 1 Amide formation by nitrilases and/or DTT[a]

Entry	Substrate	Nitrilase	DTT/mM	Time/h	Nitrile **a** (%)	Amide **b** (%)	Acid **c** (%)
1	(±)-**1a**	NIT-102	2	18	58	42	1
2	(±)-**1a**	NIT-102	0	18	71	28	1
3	(±)-**1a**	NIT-102 spin concentrated[b]	0	18	64	36	0
4	(±)-**1a**	NIT-106	0	2	4	93	3
5	(±)-**1a**	NIT-106 spin concentrated[b]	0	2	19	75	6
6	(±)-**1a**	—	0	18	100	0	0
7	(±)-**1a**	—	2	18	45	55	0
8	(±)-**2a**	—	2	18	47	53	0
9	(±)-**2a**	NIT-106	0	2	44	56	0
10	(±)-**2a**	NIT-106 spin concentrated[b]	0	2	72	28	0

[a] Values are the average of 2–5 experiments. [b] Low molecular mass components separated by repeated ultrafiltration.

Notes and references

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8 The reactions proceeded at 30 °C and 1100 rpm in an Eppendorf Thermomixer. After the scheduled time the products were isolated by extracting twice with 250 μL of ethyl acetate, respectively. Conversions were determined by RP-18 HPLC with a solvent gradient of acetonitrile/0.1% H_3PO_4 and a flow of 0.8 ml min^{-1} (30/70 from 0–2 min, 80/20 from 10–15 min and 30/70 from 15–23 min). The values are calculated from the UV response at 254 nm as area normalization percentage and averaged over 2 to 5 measurements.

9 It should be stressed at this point that a radical mechanism cannot be ruled out. Such a mechanism would comprise a single electron transfer from a sulfhydryl-group to the nitrile, which is subsequently quenched by water to give the amide.

10 The reaction conditions for mandelonitrile **3a** were chosen on a more preparative level: to 900 μL of 50 mM citrate buffer (pH 6.03) or K-phosphate buffer (pH 7.00 and 8.00) containing 1 mM EDTA and 50 mM DTT 100 μL of substrate solution (200 mM in MeOH) were added to give a final concentration of 20 mM. The reactions proceeded at 30 °C and 1100 rpm in an Eppendorf Thermomixer. At intervals samples were drawn and mixed with the same volume of HCl (0.1 N). Conversions were determined by RP-18 HPLC with a solvent gradient acetonitrile/0.1% H_3PO_4 and a flow of 1.0 ml min^{-1} (5/95 from 0–10 min, 50/50 from 15–20 min and 5/95 from 20–25 min). The values are calculated from the UV response at 225 nm using external calibration and averaged over 2 measurements.

11 *Protein Purification Handbook*, Amersham Biosciences, 2001, ch. 8, pp. 60–70.

12 Data not presented.