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Cytochrome P-450-dependent catabolism of triethanolamine in *Rhodotorula mucilaginosa*

A.N. Fattakhova*, E.N. Ofitserov & A.V. Garusov
The Kazan State University, Kazan 420008 USSR (requests for offprints)*

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Abstract

The yeast *Rhodotorula mucilaginosa* was able to grow in media containing triethanolamine or diethanolamine as the sole nitrogen source. During growth in the presence of triethanolamine, extracts of yeast cells contained increased levels of cytochrome P-450 dependent monooxygenase which catalyzed the oxidative N-dealkylation of aminoalcohols. Formation of diethanolamine, ethanolamine and glyoxylate from triethanolamine was demonstrated, and the identity of the products was verified by thin layer chromatography. These observations suggested the following scheme of triethanolamine catabolism: triethanolamine → diethanolamine + glycolaldehyde, diethanolamine → ethanolamine + glycolaldehyde, ethanolamine → NH_3 + glycolaldehyde → glycolate → glyoxylate → glycerate pathway.

Introduction

Triethanolamine and diethanolamine are compounds of potential environmental concern because they are present in a number of industrial and consumer products, including detergents, cosmetics, medicines, shampoos and cutting fluids (Bonfanti et al. 1985; Loeppky et al. 1985; Bennett 1986).

Triethanolamine (TEA) is a component of some biologically active xenobiotics, such as silatranes and germanotranes, which are cyclic organosilicon and organogermanium tris(2-oxalkyl) amino esters. The possibility of their wide application as efficient plant and animal stimulators (Voronkov et al. 1982) generated a need for an extensive study of their metabolism in microorganisms. Our previous studies have shown that the imperfect yeast *Rhodotorula mucilaginosa* could utilize the organosilicon ester of TEA as sole nitrogen source (Fattakhova et al. 1987), and the aminoalcohol was a metabolite (results unpublished). Probably, the biodegradation of these xenobiotics depends on the possibility of yeast to utilize TEA as growth substrate.

Degradation of TEA via triethanolamine-N-oxide and diethanolamine (DEA) was reported for Gram-negative bacteria grown in media containing 1 g/l TEA as the sole source of carbon and energy (Williams & Callely 1982). DEA and its derivatives were oxidized by a bacterium isolated from industrial cutting fluids (Gannon et al. 1978). However, the metabolic pathway of TEA degradation in yeasts has not yet been clarified.

According to Green & Large (1983), timethylamine and dimethylamine (as TEA and DEA analogues) catabolism in methazotrophic yeasts was repressed by CO.

Cytochrome P-450 is known to catalyze oxidation of n-alkanes, ethanol, and other compounds in yeasts and fungi (Krauzova & Sharyshev 1987; Shoun et al. 1989).

The aim of the present work to elucidate the pathway of the TEA utilization in the yeast *Rhodotorula mucilaginosa* and to study the role of cytochrome P-450 in the degradation of this aminoalcohol.

Materials and methods

Microorganism and growth conditions

The yeast strain *Rhodotorula mucilaginosa* BKIIM Y-706 was isolated as described previously (Fattakhova et al. 1987). Cells were grown on a shaker (120 rpm) at 30° C in a mineral medium described by Fattakhova et al. (1987), with 1 mM glucose plus 4.5 or 9 mM aminoalcohols.

The cell biomass was followed by measuring the culture absorbance (1 cm cuvette, 660 nm).

Chemicals

Triethanolamine was obtained from Serva Feinbiochemica GmbH & Co (Heidelberg, Germany). Cuprizone was from Fluka Chemie AG (Buchs, Switzerland). All other chemicals unmarked in the text were purchased from Sigma Chemical Co (St. Louis, USA).

Preparation of cell extracts

Cells were harvested, washed twice with distilled water and destroyed by French press at a pressure of 120 MPa at 0° C. Homogenates were centrifuged at 20 000 g for 40 min and the resulting supernatants were used for enzyme assay.

Enzyme assays

Aminoalcohol oxygenases, glycolaldehyde dehydrogenase, and tartronic semialdehyde reductase were measured as described (Williams & Callely 1982). Ethanolamine oxidase, and catalase were determined as described by Haywood and Large (1981). Glycolate oxidoreductase activity (EC 1.1.3.1) was measured spectrophotometrically (Lord 1972). Isocitrate lyase was analyzed as described by Dixon & Kornberg (1959). The glyoxylate dehydrogenase (EC 1.2.1.17) was measured according to Quayle & Taylor (1961). Glyoxylate reductase (EC 1.1.1.26) was measured according to the method of Ornston & Ornston (1969). Protein was determined by the method of Lowry et al. (1951).

Purification of cytochrome P-450

Ammonium sulfate was added to the crude extract (80% saturation). The sediment was dissolved in 10 mM phosphate buffer, pH 6.0, containing 1 mM EDTA and 1 mM dithiothreitol, and the solution was dialyzated against 10 volumes of the same mixture plus 10 BDM FAD at 0° C. The dialyzate was fractionated in a mixture of Ficoll 400, dextran, and polyethylene glycol 2000 (Sadler et al. 1985; Karenlampi et al. 1986). The fraction containing cytochrome P-450 was applied to a Sephadex G-75 column. Cytochrome P-450 was eluted with 10 mM phosphate buffer. The flow rate was 40 ml/h.

Cytochrome P-450 was assayed by reduced CO-difference spectrophotometry as described by Omura & Sato (1964) using a 'Specord UV-VIS' spectrophotometer (Karl Zeiss, Jena, Germany). The concentration of cytochrome P-450 in fractions was calculated using $\varepsilon = 91\,mM^{-1}\,cm^{-1}$ for the enzyme. TEA-oxygenase activity was also measured in those fractions.

Thin layer chromatography

To determine aminoalcohols, chloroform extracts of cultural fluid of the TEA grown yeast was analyzed on Silufol UV-254 plates (0.25 mm thick; Kavalier, Brno, Czechoslovakia). Plates were developed using the following solvent systems (v/v): (a) ethanol: 25% ammonia in water (80 : 20); (b) isopropanone: 25% ammonia in water: water (80 : 10 : 10); (c) ethanol : diethyl ether: 25% in water (32 : 10 : 1). The developed chromatograms

were visualized by spraying with Fast Blue B Salt reagent (0.5% aqueous solution of tetrazolized o-dianizidine) and with 0.1 N NaOH, followed by heating. Under this condition aminoalcohols appeared as light to dark orange-red spots.

To determine glycoaldehyde we analyzed reaction mixtures containing extracts of TEA grown yeast in 30 ml 100 mM phosphate buffer, pH 8.0 with 100 μM aminoalcohol and, in similar experiments with TEA and DEA, 15 BDM NADH. Mixtures were incubated for 30 min at 30° C. To determine glyoxylate, extracts of ethanolamine (MEA) grown cells was placed in 100 ml of 50 mM phosphate buffer, pH 8.0, containing 350 μM sodium glycolate and 90 μM phenazine methosulfate. The mixture was incubated for 10 min at 30° C. Then all reaction mixtures were incubated with 2.4-dinitrophenyl hydrazine to produce hydrazones (Walker & Taylor 1983). TLC analysis of diethyl ether extracts of the reaction mixtures was performed on Silufol UV-254 plates using the following solvent systems (v/v): (d) benzene : dioxane (70 : 20); (e) benzene : dioxane (90 : 20); (f) chloroform : dioxane : acetic acid (30 : 2,6 : 1); (g) benzene : dioxane : acetic acid (60 : 36 : 4).

Determination of triethanolamine by gas liquid chromatography

NaOH was added, to pH 10, to 5 ml of cultural fluid which was then evaporated at 60° C. The dry residue was silylated with 100 μl of hexamethyl silazane for 20 min, then 1 ml of acetone was added. Samples were analyzed on a Chromatone N-AW-DMCS column with 10% SCTPV-803 (PO Orgsintez, Kazan USSR). The temperature of the column was 170° C, that of the evaporator was 230° C. TEA peak time was 15 min.

Table 1. Identification of metabolites formed by *Rhodotorula mucilaginosa* cells grown on triethanolamine by thin-layer chromatography.

Components	R_f values in solvents		
	a	b	c
Triethanolamine (standard)	0.37	0.64	0.48
Component 1	0.37	0.64	0.47
Diethanolamine (standard)	0.32	0.23	0.45
Component 2	0.33	0.23	0.44
Monoethanolamine (standard)	0.21	0.20	0.37
Component 3	0.22	0.19	0.37

Ammonia analysis

Ammonium ions were determined by titration (Lurye 1984).

Results

The yeast *Rhodotorula mucilaginosa* used TEA as sole nitrogen source. During growth, the yeast degraded 550 mg/l TEA. TLC analysis of the yeast growth medium with TEA as a substrate revealed 2 metabolites identified as DEA and MEA (Table 1). The yeast could utilize those aminoalcohols as sole nitrogen sources. DEA proved to be the most toxic for the *Rh. mucilaginosa* in comparison with MEA and TEA at 4.5 mM. If the concentrations of aminoalcohols in the growth media were increased up to 9 mM they all inhibited growth (results not shown).

According to the above data we assumed participation of NAD(P)H-dependent oxygenases in aminoalcohol catabolism. Table 2 shows the data providing that the enzymes responsible for the oxidation of TEA and DEA were inducible. The comparison of oxidation with NADPH or NADH in the presence of aminoalcohols showed that the latter cofactor was the best hydrogen donor. FAD increased NADH oxidation by about 20% (Table 2).

Ammonium (15 mM) was a strong repressor of TEA and DEA oxygenase synthesis, when added to the induction mixture along with TEA. The TEA-oxygenase and DEA-oxygenase activities were found to decrease by 82.6% in the presence of exogenic ammonia (23.3 and 15.5 nmol/min per mg of protein respectively).

Contact of yeast cell-free extracts with CO during 20 sec caused 100% inhibition of TEA oxygenase. It thus became of interest to determine cyto-

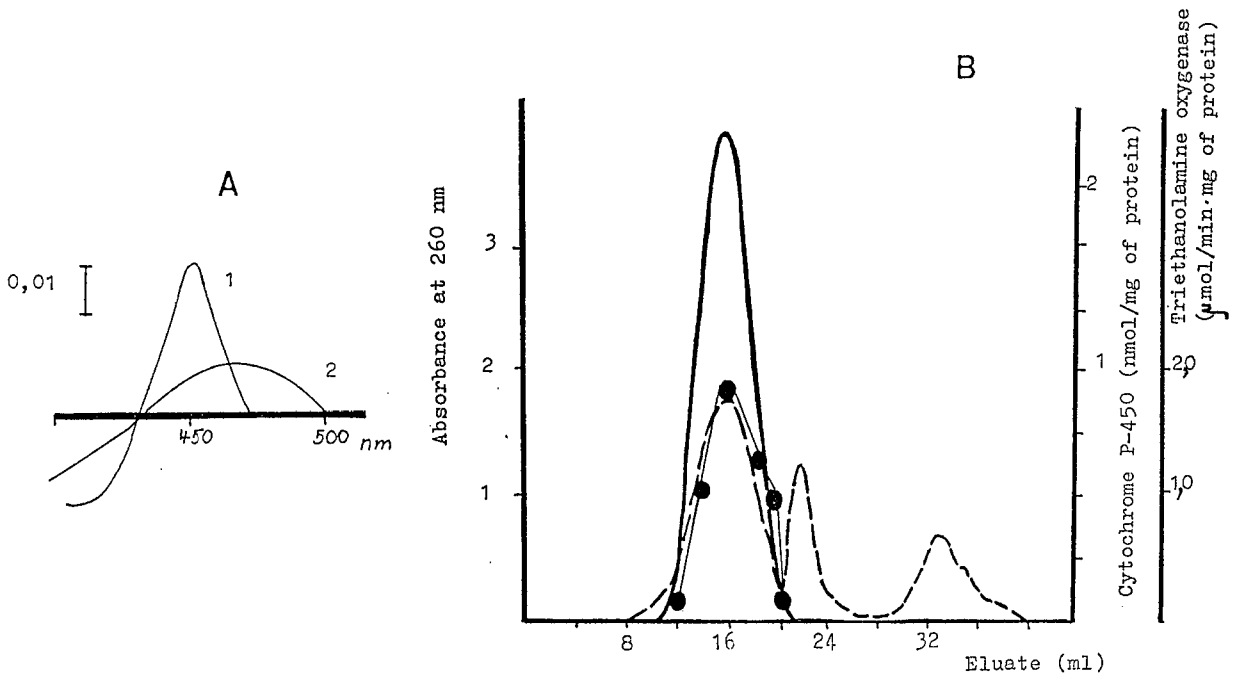

Fig. 1A. CO-difference spectra of cell-free extracts of *Rhodotorula mucilaginosa* grown on triethanolamine (1) or on ammonium sulfate (2). 1B. Elution profile for gel chromatography of fraction containing cytochrome P-450. The Sephadex G-75 column was eluted at 40 ml/h and fractions (4 ml) were collected. (———) cytochrome P-450; (—) absorbance at 260 nm; (.●.) triethanolamine – oxygenase activity.

chrome P-450 levels in cells as a function of nitrogen source. For the same purpose we compared CO-difference spectra of cell-free extracts of *Rh. mucilaginosa* grown with ammonium sulfate and TEA. TEA grown yeast cells contained 0.6 nmol of cytochrome P-450 per mg of protein, while ammonia grown cells did not (Fig. 1A).

To determine the activity of yeast cytochrome P-450, cell protein was partly purified by fractionation and gel filtration. We achieve 10-fold enrich-

Table 2. Activities of triethanolamine oxygenase and diethanolamine oxygenase in cell-free extracts of *Rhodotorula mucilaginosa* grown on various nitrogen sources.

Enzyme + cofactor	Specific activities [nmol/min (mg protein)] Nitrogen source for growth	
	Triethanolamine	Ammonium sulfate
TEA-oxygenase (NADH)	131.8	2.0
TEA-oxygenase (NADH + FAD)*	152.5	2.0
TEA-oxygenase (NADPH)	45.6	0
DEA-oxygenase (NADH)	90.2	0
DEA-oxygenase (NADH + FAD)*	126.4	2.0
DEA-oxygenase (NADPH)	12.5	0
NADH oxidase	2.5	2.0

* FAD (15 μM) was added to cell-free extracts.

ment of cytochrome P-450 in the enzyme preparation. The comparison of cytochrome content and TEA-oxygenase activity in cell protein fractions suggested that, in the yeast, oxidative N-dealkylation of TEA was catalyzed by a cytochrome P-450 dependent TEA-oxygenase (Fig. 1B). TEA-oxygenase activity in those fractions was completely inhibited by CO, sodium azide (0.1 mM), N-ethylmaleimide (1.5 mM) and n-octylamine (1 mM).

The glycolaldehyde thus formed as the result of TEA, DEA and MEA oxidation was demonstrated by TLC analysis of reaction mixtures containing the aminoalcohol, cell-free extract, and NADH for TEA and DEA oxygenase. The R_f values for hydrazone glycolaldehyde in the d, e, f systems were 0.75, 0.63 and 0.50 respectively. No glycoaldehyde or acetaldehyde accumulated in the medium during growth on aminoalcohols.

Growth on TEA, DEA or MEA was accompanied by the induction of ethanolamine oxidase catalyzing the oxidative deamination of MEA (Table 3). Nitrogen was liberated as ammonia (Fig. 2).

Exogenic ammonia (10 mM) was a strong repressor of MEA synthesis. Formation of glycolaldehyde and ammonia (Fig. 2) from MEA, and induction of catalase activity in cells grown on TEA (Table 3) or on MEA (results not shown) allowed to suggest an oxidase enzyme to be involved in MEA catabolism since acetaldehyde is known to be

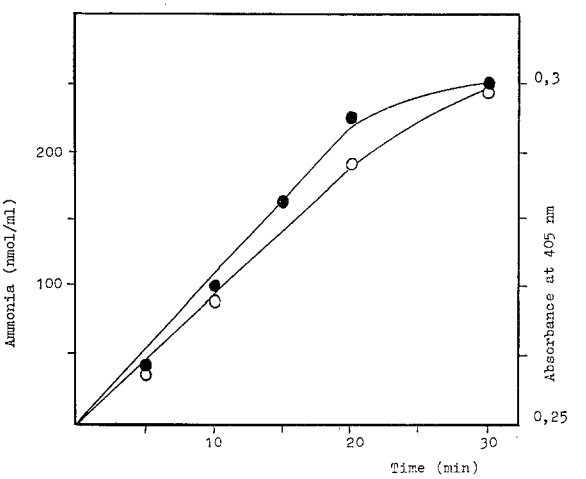

Fig. 2. Ammonia formation in ethanolamine oxidase reaction. (●) ammonia; (○) absorbance at 405 nm.

a product of the alternative lyase reaction (Zwart et al. 1983).

MEA oxidase of *Rh. mucilaginosa* was inhibited by copper-chelating agents, sodium azide, and hydrazine derivatives (Table 4) like typical yeast amine oxidase (Haywood & Large 1981).

Formation of glyoxylate in the reaction mixtures with glycolate, cofactors and cell-free extracts of yeast grown on TEA, was detected on TLC. R_f values for hydrazone glyoxylate in the e, f, g systems were 0.30, 0.60 and 0.89 respectively.

Consideration of possible routes from MEA to

Table 3. Specific activities of enzymes likely to be involved in the catabolism of ethanolamine ion *Rhodotorula mucilaginosa.*

Enzymes	Specific activity [nmol/min (mg protein)] Nitrogen source for growth:	
	Ammonium sulfate	Triethanolamine
1. Ethanolamine oxidase	0	110.5
2. Catalase*	10.1	35.2
3. Glycolaldehyde dehydrogenase	0	161.8
4. Glycolate oxidoreductase	0	81.1
5. Tartronic acid semialdehyde reductase	10.8	40.6
6. Glyoxylate dehydrogenase	0	0
7. Glyoxylate reductase	0	0
8. Isocitrate lyase	4.5	3.2
9. NADH oxidase	2.0	2.0

* Catalase activity [μmol/min (mg protein)].

glyoxylate led to search in yeast cell-free extracts for enzymes capable of producing glyoxylate. Activities analogous to glycolaldehyde dehydrogenase (Williams & Callely 1982) and glycolate oxidoreductase (Lord 1972) were detected in extracts of the yeast grown on TEA. Activities of glyoxylate dehydrogenase, glycolate reductase and isocitrate lyase could not be found. In contrast, an enzyme analogous to tartronic semialdehyde reductase (Williams & Callely 1982) was highly active (Table 3).

Discussion

Growth of *Rh. mucilaginosa* on TEA as a sole source of nitrogen was found to result in transient production of DEA, MEA and glycolaldehyde. The enzymes of TEA catabolism, TEA-oxygenase, DEA-oxygenase and MEA oxidase, were inducible. Probably, DEA catabolism in *Rh. mucilaginosa* was catalyzed by an oxygenase analogous to that in a bacterium isolated by Williams & Callely (1982). In contrast, in yeast TEA was oxidized directly to DEA without TEA-N-oxide formation. However, FAD increased TEA-oxygenase activity by about 20%, FAD dependent oxygenases could hardly take part in the aminoalcohol catabolism. The cofactor was used to preserve the enzyme in the cell-free extracts as in *Candida boidinii* grown on alkylamines (Green & Large 1983). The activity of FAD dependent trimethylamine oxygenases in *Sporopachydermia cereana* increased 5 times in the presence of FAD (Whitefield & Large 1986). NADH was the best hydrogen donor for oxygenation of aminoalcohols, similar as in C-hydroxylation of alkylamines in *C. boidinii* (Green & Large 1983).

100% of CO inhibition of TEA catabolism was typical of *Rh. mucilaginosa*. Analysis of CO-difference spectra of cell-free extracts showed that growth with TEA was accompanied by induction of cytochrome P-450 in cells. We could not identify cytochrome P-450 in yeast cells grown with ammonium sulfate (Fig. 1A). It should be noted that, according to Sanglard et al. (1984), the cytochrome P-450 identification in crude cell extracts by the method of Omura and Sato (1964) can give false-negative results when cells were grown with glucose. Cytochrome P-450 was induced in *Rh. mucilaginosa* cells grown aerobically with TEA and glucose, similar to *Saccharomyces cerevisiae* grown on ethanol and glucose (Blatiak et al. 1985).

Comparing the cytochrome P-450 with TEA-oxygenase in cell protein fractions (Fig. 1B), we may confirm, that cytochrome P-450 dependent monooxygenase catalyze the oxidative N-dealkylation of aminoalcohol:

$$(OHCH_2CH_2)_3N \xrightarrow[NAD(P)H \quad NAD(P)+]{O_2 \quad H_2O} (OHCH_2CH_2)_2\,NH + CH_2(OH)CHO$$

DEA is likely to be converted to MEA and glycolaldehyde in a similar scheme, because DEA-oxygenase was inhibited by CO.

MEA catabolism in methazotrophic yeasts is known to proceed by direct oxidation of the aminoalcohol by a specific amine oxidase (Zwart et al. 1983). The data obtained prove that in *Rh. mucilaginosa* the inducible copper-binding ethanolamine oxidase catalyzed oxidative deamination of MEA. The activity of aminoalcohol oxidation (Table 3) was 5 times higher than that in methazotrophic yeasts (Zwart et al. 1983).

The synthesis of MEA oxidase was repressed by ammonia. According to Zwart & Harder (1983), MEA oxidase 'keeps the role as a major channel' through which all nitrogen that is metabolized must

Table 4. Effect of various inhibitors on ethanolamine oxidase activity in cell-free extracts of *Rhodotorula mucilaginosa* grown on triethanolamine.

Inhibitor	Concentration (mM)	Percentage inhibition of activity
Hydrazine sulfate	1	100
Hydroxyammonium chloride	1	100
Sodium azide	0.01	100
EDTA	1	92
EDTA + Cu^{2+}	1	0
EDTA + Zn^{2+}	10	96
EDTA + Ca^{2+}	10	100
EDTA + Mo^{2+}	1	90
EDTA + Mn^{2+}	1	93
Cuprizone	0.001	100

pass. Therefore, such a regulation may be a reason of the observed uncapability of *Rh. mucilaginosa* to utilize the aminoalcohols also as sole carbon source.

Probably, TEA catabolism in the yeast grown on ammonium sulfate and aminoalcohol was inhibited in TEA and DEA N-dealkylation by nitrogen catabolite repression since oxygenase synthesis was reduced 4–6 times at the presence of exogenic ammonia.

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