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Characterization of an inducible, membrane-bound iminodiacetate dehydrogenase from *Chelatobacter heintzii* ATCC 29600

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Abstract

Iminodiacetate (IDA) is a xenobiotic intermediate common to both aerobic and anaerobic metabolism of nitrilotriacetate (NTA). It is formed by either NTA monooxygenase or NTA dehydrogenase. In this paper the detection and characterization of a membrane-bound iminodiacete dehydrogenase (IDA-DH) from *Chelatobacter heintzii* ATCC 29600 is reported, which oxidizes IDA to glycine and glyoxylate. Out of 15 compounds tested, IDA was the only substrate for the enzyme. Optimum activity of IDA-DH was found at pH 8.5 and 25° C, respectively, and the K_m for IDA was found to be 8 mM. Activity of the membrane-bound enzyme was inhibited by KCN, antimycine and dibromomethylisopropyl-benzoquinone. When inhibited by KCN IDA-DH was able to reduce the artificial electron acceptor iodonitrotetrazolium (INT). It was possible to extract IDA-DH from the membranes with 2% cholate, to reconstitute the enzyme into soybean phospholipid vesicles and to obtain IDA-DH activity (more than 50% recovery) using ubiquinone Q_1 as the intermediate electron carrier and INT as the final electron acceptor. Growth experiments with different substrates revealed that in all NTA-degrading strains tested both NTA monooxygenase and IDA-DH were only expressed when the cells were grown on NTA or IDA. Furthermore, in *Cb. heintzii* ATCC 29600 growing exponentially on succinate and ammonia, addition of 0.4 g l^{-1} NTA led to the induction of the two enzymes within an hour and NTA was utilized simultaneously with succinate. The presence of IDA-DH was confirmed in ten different NTA-degrading strains belonging to three different genera.

Abbreviations: cA – component A; cB – component B; DBMIB – dibromomethylisopropyl-benzoquinone; HEPES – hydroxyethylpiperazinethanesulfonic acid; IDA – iminodiacetate, $HN(CH_2COOH)_2$; IDA-DH – iminodiacetate dehydrogenase; INT – iodonitrotetrazolium chloride; NTA – nitrilotriacetate, $N(CH_2COOH)_3$; NTA-MO – nitrilotriacetate monooxygenase; PMS – phenazine methosulphate; SDS-PAGE – sodium dodecylsulfate polyacrylamide gel electrophoresis; Suc-DH – succinate dehydrogenase

Introduction

The complexing agent nitrilotriacetate (NTA) is used for a range of different purposes one of which is as a substitute for sodium triphosphate in laundry detergents (Tiedje 1980). Many obligately aerobic and facultatively denitrifying microorganisms have been isolated which can use NTA as a sole source of

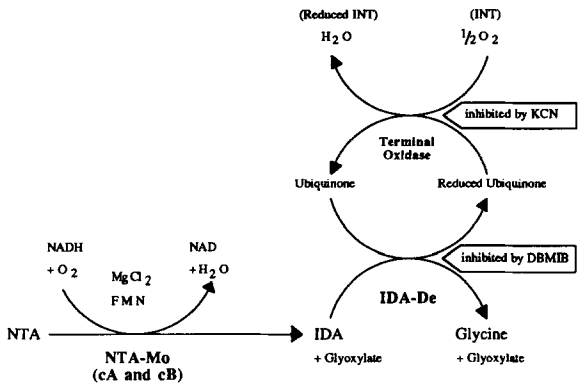

Fig. 1. Metabolic pathway of NTA catalysed by NTA-MO and the membrane-bound IDA-dehydrogenase (IDA-DH) in *Chelatobacter heintzii* ATCC 29600.

nitrogen, carbon and energy. The majority of such isolates are Gram-negative, obligately aerobic rods (Cripps & Noble 1973; Egli et al. 1988; Focht & Joseph 1971; Kaki et al. 1986; Tiedje et al. 1973) which initially had been identified as *Pseudomonas* spp.. Recently it has been shown that these obligately aerobic isolates belong to two new genera (obligately aerobic) for which the names *Chelatobacter* (*heintzii*) and *Chelatococcus* (*asaccharovorans*) have been proposed (Auling et al. 1993).

The biochemical pathway for NTA degradation was first investigated in the two virtually identical strains of *Cb. heintzii* ATCC 29600 and isolate T23 (Cripps & Noble 1973; Firestone & Tiedje 1978). In both strains a monooxygenase (NTA-MO) was reported to be responsible for the oxidative conversion of NTA to iminodiacetate (IDA) and glyoxylate (Fig. 1). Recently, it was possible to purify a functional NTA-MO to homogeneity from *Cb. heintzii* ATCC 29600 (Uetz et al. 1992). The enzyme consisted of an NADH-oxidizing component (cB) and a second component cA with unknown function but necessary for the conversion of NTA to IDA and glyoxylate. The substrate specificity of NTA-MO was restricted to NTA. IDA, which was proposed to be a substrate for the NTA-MO as well (Firestone & Tiedje 1978), was not turned over by the purified enzyme. In this paper the detection of a membrane-bound IDA dehydrogenase (IDA-DH) which converts IDA to glycine and glyoxylate is reported. Furthermore, the presence and the regulation of both NTA-MO and IDA-DH in different NTA-utilizing strains has been investigated.

Materials and methods

Bacterial strains and growth conditions. Cb. heintzii strains ATCC 29600 and ATCC 27109 were obtained from the American Type Culture Collection, Rockville, Md.. Isolates TE2 and TE4–TE11 were isolated in our laboratory and all strains were maintained on a synthetic medium containing 1 g l^{-1} NTA as described previously (Egli et al. 1988). In order to avoid excretion of large amounts of ammonia during large scale growth (100l) the bacteria were grown on a mixture of NTA and acetate (1 g l^{-1}, each). Towards the end of the exponential phase additional NTA and acetate (1 g l^{-1}, each) was added to the culture and the pH was kept in the range of 6.5 to 7.5 by discontinuous addition of 0.5 M orthophosphoric acid. Cells were harvested by centrifugation at an optical density at 600 nm of 1.2 and the cell paste (350 g) was frozen at −70° C.

Batch growth of strain ATCC 29600 on different substrates and growth of different NTA-degrading strains on NTA was carried out in 11 Erlenmeyer flasks containing 500 ml of medium. The medium contained 1 g l^{-1} substrate and 0.6 g l^{-1} of Na-acetate. Media containing 1.5 g l^{-1} succinate or citrate, respectively, were supplemented with 1 g l^{-1} of NH_4Cl as a nitrogen source. Otherwise, the composition of the media were as described previously (Egli et al. 1988). Cells were harvested at the end of the exponential phase by centrifugation at 8000 g for 10 min, resuspended in 10 ml 20 mM Tris-H_2SO_4 pH 8.0 and stored at −70° C.

Preparation of cell-free extract. 60 g wet weight of frozen cell paste of *Cb. heintzii* strain ATCC 29600 was suspended in 400 ml Tris-HCl buffer pH 8.0 containing 2 mM dithiothreitol, 10 mg desoxyribonuclease I, 5 mM $MgSO_4$ and the protease inhibitors leupeptin (1 μM), pepstatin (1 μM) and phenylmethanesulfonylfluoride (0.5 mM) (Keesey 1987). After homogenization for 1 minute in a Sorvall homogenizer, the cells were broken by a single passage through an Aminco French press (Aminco,

Urbana, II.) at 100 MPa. EDTA was added to a final concentration of 10 mM and the suspension was centrifuged for 30 minutes at 40000 g to remove unbroken cells and cell debris. Cells from the 0.5 l batch cultures were broken after thawing and homogenization by a single passage through the French press in the presence of $MgSO_4$ and desoxyribonuclease but without addition of DTT and protease inhibitors. Unbroken cells and cell debris were removed by centrifugation for 10 min at 12000 g.

Preparation of membrane vesicles. The cell-free extract was centrifuged for 2 hours at 160000 g, the supernatant (cytosolic fraction) was used for determination of NTA-MO activity. The pellet consisted of the membrane vesicles. In order to remove cytosolic proteins included in the membrane vesicles as well as the ribosomes the pellet was resuspended in 200 ml 50 mM Tris-H_2SO_4 pH 8.0, 10% sucrose, 5 mM EDTA by sonication and collected by centrifugation for 2 hours at 160000 g. The supernatant was discarded and the pellet was resuspended by brief sonication of 20 ml 50 mM Tris-H_2SO_4 pH 8.0 and the washed membrane vesicles obtained by this procedure were stored in 1 ml portions at −70° C. The membrane pellets, obtained from the 160000 g centrifugation of cell-free extracts from cells grown in shake flask batch cultures, were directly resuspended in 1 ml 50 mM Tris-H_2SO_4 pH 8.0 and used for experiments without applying the washing procedure.

Enzyme assays

i) NTA monooxygenase assay. NTA-MO was assayed as described previously measuring the consumption of NTA in cytosolic fractions in the presence of $MgCl_2$, FMN and NADH (Uetz et al. 1992).

ii) Assay for membrane-bound dehydrogenases by oxygen consumption. Activity was determined in a closed reaction vessel (0.8 ml total volume) fitted with a Clarke type oxygen electrode (Yellow Springs Instruments, Yellow Springs, Ohio) at 20° C if not otherwise stated. Membrane vesicles in 50 mM Tris-H_2SO_4 pH 8.0, containing 1–4 mg protein per ml, were placed in the reaction vessel and the reaction was started by addition of the substrate. The decrease in oxygen concentration was recorded and the substrate dependent oxygen consumption rate was determined using a value for the concentration of O_2 (air saturated) of 0.288 mM at 20° C (Anonymous 1928). As only one atom of oxygen is consumed per substrate molecule used (Fig. 1, Table 1) the value obtained was multiplied by 2 and the activity expressed as nmol substrate used per min.

iii) Assay for IDA-DH via reduction of INT. The assay was performed in 1 ml cuvettes, containing 50 mM Tris-H_2SO_4 pH 8, 0.2 mM INT, 1 mM Ubiqui-

Table 1. Stoichiometry of the reaction catalysed by the washed membrane fraction prepared from NTA-grown *Cb. heintzii* ATCC 29600.[a]

IDA concn (mM)	Time (min)	IDA consumed (mM)	O_2 consumed (mM)	INT reduced (mM)	Glyoxylate produced (mM)	Glycine produced (mM)
10	4.5	n.d.	0.26		0.53	0.55
10	9	n.d.	n.d.		0.94	1.12
10	14	n.d.	n.d.		1.52	1.56
2	0	0	0		0.01	0.01
2	10	0.54	0.23		0.50	0.42
2[b]	10	0.41	0	0.82	n.d.	0.46

[a] The reaction vessel contained washed membranes (0.65 mg protein ml^{-1}) in 50 mM ammonium acetate at pH 8.0. In a control experiment containing protein denatured with heat (10 min at 95° C) neither IDA nor oxygen was consumed and neither glyoxylate nor glycine was formed.

[b] The reaction was carried out in the presence of 1 mM KCN.

n.d. Not determined.

none Q_1, 50mM $CaCl_2$ and 42mM IDA (from a stock solution of 0.84M at pH 9.5). Approximately 0.1mg protein was added to start the reaction and the change in absorption at 500nm was recorded against a reference cuvette containing all ingredients except IDA. No reduction of INT was observed in the reference cuvette. Activity was calculated as nmol INT reduced per min using a molar extinction coefficient of reduced INT ε_{500} = 11100 cm^{-1} mol^{-1}, which was determined by the complete reduction of 0.1mM INT with 1mM DTT.

Analysis of NTA and succinate. To measure NTA in cell-free extracts TCA was added to samples to a final concentration of 5% (v/v) and precipitated protein was removed by centrifugation for 5min at 10000g. For the determination of substrate concentrations in growing cultures, the cells were removed from 1ml samples by centrifugation for 2min at 15000g and dilution of the supernatant 1 to 10 in water before analyzing for NTA and succinate by high pressure ion exclusion chromatography as described by Schneider et al. (1988).

Analysis of IDA. IDA was determined by a modified procedure of Schneider et al. (1989). The sample (100µg) was mixed with 200µl 30mM H_3PO_4, boiled for 5min and the precipitated protein was removed by centrifugation. The supernatant (215µl) was mixed with 500µl H_2O before analysis. Separation was achieved on a Dionex AS4A column using 2mM tyrosine in 4.5mM NaOH as the eluent with a Dionex 2000i high pressure chromatography system connected to a Dionex CDM2 conductivity detector (Dionex, Sunnyvale, CA).

Analysis of glyoxylate. Prior to analysis of glyoxylate, samples of 100µl were mixed with 40µl of 67mM HCl to stop IDA-DH activity. The amount of glyoxylate in the sample was analyzed with the phenylhydrazine/$K_3Fe(CN)_6$ method described by Trijbels & Vogels (1966), but using a total assay volume of 280µl.

Analysis of glycine. Prior to analysis, samples of 100µl were mixed with an equal volume of 2% (v/v) trifluoracetic acid and precipitated protein was removed by centrifugation for 2min at 15000g. A 100µl aliquot of the supernatant was freeze dried to remove volatile compounds, redissolved in 1ml Nanopur water and analysed for glycine as described by Knecht & Young (1986).

Protein determination. Protein concentrations were measured using the BCA (Bicinchoninic acid) protein assay (Pierce, Rockford, IL). Bovine serum albumin was used as the standard. For determination of protein concentration during solubilization and reconstitution of IDA-DH, a modified Lowry method was used (Peterson 1979).

Solubilization of IDA-DH. To 2.4ml of a suspension of membrane vesicles in 50mM Tris-H_2SO_4 pH 8.0, containing 10–20mg protein per ml and 1mM phenylmethanesulfonylfluoride, 0.8ml of 50% glycerol (v/v) was added. Whilst the suspension was stirred at 4°C 0.8ml of a 10% (w/v) Na-cholate solution containing 2% soybean phospholipids, was added dropwise to give a final concentration of 2% cholate. After 20min stirring at 4°C, the suspension was centrifuged for 50min at 250000g and the supernatant was referred to as solubilized membrane proteins. Solubilization of IDA-DH activity was unsuccessful with the following detergents: 1% Lubrol, 1% octyl-PoE, 1% deoxycholate, 1% Triton-X-100, 1% CHAPS (3-(3-cholamidopropyl)dimethyl-ammonio-1-propane sulfonate), 0.4% dodecyl maltoside and 1% octylglucoside.

Reconstitution of IDA-DH activity in artificial soybean phospholipid vesicles. A modified procedure described by Varadhachary & Maloney (1990) was used to reconstitute IDA-DH from the solubilized membrane protein fraction in artificial soybean phospholipid vesicles. A suspension of 4% (w/v) soybean phospholipids in 2% Na-cholate was prepared by sonication and 1ml thereof was mixed with 3ml of the solubilized membrane protein fraction. After 20min gentle shaking at 4°C followed by 30s of mild sonication the mixture was rapidly diluted by addition of 80ml of cold 50mM Tris-H_2SO_4 (pH 8.0) solution. After 30min the slightly turbid sus-

pension was centrifuged for 2h at 160000g and the pellet was resuspended in 2ml 50mM Tris-H_2SO_4 pH 8.0 by brief sonication.

Preparation of antisera. Antisera against cA and cB of NTA-MO from *Cb. heintzii* ATCC 29600 were prepared as described previously (Uetz et al. 1992). Specificity of the two antisera was tested by using the immunoblotting technique described below. None of the sera reacted against proteins of cell-free extracts from succinate-grown *Cb. heintzii* ATCC 296000 (T. Uetz 1992).

Immunodetection of the two NTA-MO components. Protein samples were run on 12% SDS-PAGE gels (Laemmli 1970) and blotted onto a nitrocellulose sheet following the method of Towbin et al. (1979). The nitrocellulose sheet was incubated in 20ml PBS (phosphate buffered saline; 100mM K-PO_4, pH 7.2, 150mM NaCl) containing 4% PVP (Polyvinylpyrrilidone) for 2h. Subsequently it was incubated in 20ml PBS containing 2% PVP, 10µl antiserum against cA and 10µl antiserum against cB for 1h. This was followed by extensive washing with PBS containing 2% PVP and incubation in PBS containing 2% PVP and 1µCurie of ^{125}I-labeled protein A for 45min (Harlow & Lane 1988). After washing the sheet with PBS and drying, it was exposed for 60 hours to a Fuji RX medical X-ray film.

Chemicals. Ubiquinone Q_1 was a gift from Dr. U. Fischer of Hoffmann-La Roche, Basel, Switzerland. Catalase, desoxyribonuclease I, dithiothreitol and phenylmethanesulfonylfluoride were obtained from Serva, Heidelberg, FRG. Leupeptin and pepstatin were products of Boehringer, Mannheim, FRG. N-methylated amino acids, soybean phospholipids, Antimycine, DBMIB and ubiquinone Q_{10} were supplied from Sigma, St. Louis, MO. All other chemicals were of analytical grade and were obtained from either Fluka, Buchs, Switzerland or Merck, Darmstadt, FRG.

Results

Oxidation of IDA by washed membrane preparations from Chelatobacter heintzii *ATCC 29600.* In the search for an enzyme, able to transform IDA, it was found that IDA stimulated oxygen consumption of membrane preparations obtained from NTA-grown cells of *Cb. heintzii* ATCC 29600. No IDA-stimulated oxygen consumption was observed in the soluble protein fraction from such cells. IDA not only stimulated oxygen consumption in the membrane preparation, but it was oxidized stoichiometrically to glyoxylate and glycine at the expense of half a molecule of O_2 (Table 1). When the reduction of O_2 was inhibited by KCN, or in the absence of oxygen, artificial electron acceptors such as DCPIP or INT were reduced instead of O_2 (Table 1). This suggested, that washed membrane vesicles catalysed the oxidation of IDA according to the scheme given in Fig. 1.

Specificity of IDA-DH and its biochemical distinction from the membrane-bound succinate dehydrogenase. None of the following compounds were able to significantly stimulate oxygen consumption in the membrane preparations from NTA-grown cells: glycine, glyoxylate, dimethylglycine, NTA, trimethylamine, dimethylamine, methylamine, N-methylglutamate, L-proline, L-glutamate, L-aspartate, N-hydroxyethyl-IDA, N-methyl-IDA, phenylalanine, DL-alanine, glutarate and glyphosate. N-methylalanine, N-methylphenylalanine, N-methylleucine and sarcosine stimulated oxygen consumption only up to a maximum of 2.5% of the rate observed for IDA. Succinate significantly stimulated oxygen consumption of washed membranes but, in contrast to IDA oxidation, this activity was completely inhibited by 15mM malonate. Furthermore, membranes from succinate-grown cells did not exhibit IDA oxidation, whereas succinate was oxidized at a similar rate as in membranes from NTA-grown cells (see also Table 5).

Characterization of the membrane-bound IDA-DH activity. Because IDA is able to chelate metal ions it was investigated, whether some of these ions could

stimulate IDA oxidation. Furthermore, some compounds known to interact with components of the bacterial electron transport chain were tested for their ability to inhibit IDA oxidation. Table 2 summarizes the results obtained with washed membranes from NTA-grown cells. IDA oxidation was increased in the presence of $CaCl_2$, $BaCl_2$ and $MgCl_2$. Furthermore, the K_m value for IDA was 8mM in the presence of $CaCl_2$, compared to 50mM in its absence. Other divalent cations tested inhibited oxygen consumption either slightly ($MnCl_2$ and $FeCl_3$) or completely ($ZnCl_2$, $CuCl_2$ and $NiCl_2$). EDTA did not inhibit oxygen consumption, which indicated that IDA oxidation was not strictly dependent on metal ions. IDA oxidation was very sensitive to KCN, but under these conditions rapid reduction of the artificial electron acceptor INT was possible. DBMIB, which is an ubiquinone analog and was reported to inhibit reoxidation of ubiquinones (Crofts & Wraight 1983), inhibited both IDA-stimulated oxygen consumption and IDA-dependent INT reduction (Table 2). This was also the case for antimycin A which blocks electron transfer from b-type cytochromes (Crofts et al. 1983; Crofts & Wraight 1983).

The pH optimum and the temperature optimum of the membrane-bound IDA dehydrogenase were determined to be 8.2 and 28°C, respectively. The enzyme was active in a rather broad range of pH values (50% of maximum activity at pH 6.9 and 9.2, respectively) and temperatures (50% of maximum activity at 14°C and 38°C, respectively). At pH values below 5 and above 11 activity was irreversively lost, as was the case for temperatures above 45°C.

Solubilization and reconstitution of IDA-DH activity. A range of detergents (see 'Materials and methods') was tested for extraction of IDA-dehydrogenase from washed membranes. Activity of solubilized IDA-DH was determined by following INT reduction using different possible intermediate electron acceptors (PMS, ubiquinone Q_0 and ubiquinone Q_1). Only when 1% or 2% cholate was used for solubilization and ubiquinone Q_1 (to a lesser extent ubiquinone Q_0) as an intermediate electron carrier in the assay was significant IDA-dependent INT reduction observed in the solubilized membrane protein fraction. However, the amount of solubilized activity never exceeded 10% of the observed activity in the membranes (Table 3). The extracted membranes in the pellet never contained more than 10% of the original IDA-dependent INT-reducing activity. The solubilized membrane protein fraction was devoid of IDA-stimulated oxygen consumption activity, showing that the preparation was not contaminated with intact membrane vesicles. These results suggest that, to be active, IDA-DH has to be integrated in a phospholipid bilayer. Therefore, an attempt to reconstitute IDA-DH activity in artificial soybean phospolipid vesicles, by a

Table 2. Stimulation and inhibition of the membrane-bound IDA-DH from *Cb. heintzii* ATCC 29600.

Added compound (concn in mM)[a]	Assay method[b]	Relative activity (%)
Control[c]	O_2	100
$CaCl_2$ (20)	O_2	200
$BaCl_2$ (20)	O_2	214
$MgCl_2$ (20)	O_2	141
$MnCl_2$ (20)	O_2	39
$FeCl_3$ (20)	O_2	68
$CuCl_2$ (20)	O_2	0
$NiCl_2$ (20)	O_2	5
$ZnCl_2$ (20)	O_2	0
EDTA (20)	O_2	107
$CaCl_2$ (20) + KCN (1)	O_2	0
$CaCl_2$ (20) + KCN (0.1)	O_2	8
$CaCl_2$ (20) + KCN (0.01)	O_2	23
$CaCl_2$ (20) + KCN (0.1) = Control[d]	INT	100
$CaCl_2$ (20) + Antimycine (0.5)	O_2	2
$CaCl_2$ (20) + KCN (0.1) + Antimycine (0.5)	INT	1
$CaCl_2$ (20) + DBMIB (0.5)	O_2	0
$CaCl_2$ (20) + KCN (0.1) + DBMIB (0.5)	INT	0

[a] These compounds were present in the assay in addition to those present in the control experiment. The figure in brackets gives the final concentration (mM) of the added compound.

[b] Either the oxygen consumption assay (O_2) or the INT reduction assay (INT) was used to determine the activity.

[c] The control assay contained in 1 ml of 50 mM Tris-H_2SO_4 buffer at pH 8.0, 42 mM IDA, and washed membranes (1.3 mg protein with a specific activity of 184 nmol IDA min^{-1} (mg protein)$^{-1}$ in the presence of $CaCl_2$).

[d] The control assay contained in 1 ml of 50 mM Tris-H_2SO_4 buffer at pH 8.0, 42 mM IDA, 2 mM INT and washed membranes (1.3 mg protein with a specific activity of 450 nmol INT min^{-1} (mg protein)$^{-1}$ in the presence of $CaCl_2$).

modified method described by Varadhachary & Maloney (1990) was made. The specific IDA-DH activity increased 10-fold by this treatment with a recovery of 50% (Table 3). Reconstitution of IDA-DH activity in the presence of 1mM ubiquinone-Q_{10}, which is the major ubiquinone found in this genus (Auling et al. 1993), led to a recovery of 58% of IDA-DH activity (Table 3).

Characterization of reconstituted IDA-DH activity of soybean phospholipid vesicles. Ubiquinone Q_{10}-free phospholipid vesicles containing reconstituted IDA-DH activity were devoid of IDA-stimulated oxygen consumption activity and only weak INT reduction (5%) was observed in the absence of added ubiquinone-Q_1. Whereas, when IDA-DH was reconstituted in ubiquinone-Q_{10} containing phospholipid vesicles some oxygen consumption activity, which was inhibited in the presence of KCN and considerable INT reduction activity (40%) was observed without ubiquinone-Q_1 being present in the assay. This strongly suggests that *in vivo* ubiquinone-Q_{10} acts as an electron acceptor for IDA-DH. IDA-DH reconstituted in ubiquinone-Q_{10}-free phospholipid vesicles was used for a detailed characterization of the enzyme (Table 4). Reduction of INT was stimulated by the addition of a soluble ubiquinone, either Q_0 or Q_1 (K_m value for ubiquinone Q_1 was 1mM). Other electron acceptors such as duroquinone, menadione or PMS were ineffective. IDA-DH activity was strongly inhibited by DBMIB, which blocks electron transfer to ubiquinones (Crofts & Wraight 1983), and by antimycin A, which was reported to inhibit electron transfer to

Table 3. Solubilization and reconstitution of IDA-DH from *Chelatobacter heintzii* ATCC 29600 in phospholipid vesicles.

Step	Total protein (mg)	Total activity [a]	Specific activity [b]	Recovery (%)
Washed membranes	30	13500	450	100
Remaining membranes	9.3	1270	136	8
2% Cholate extract	17.8	940	53	7
Reconstituted IDA-DH	8.4	4500	540	38
Reconstituted IDA-DH (+Q_{10})[c]	8.6	6840	795	58

[a](Specific) activity was expressed as nmol INT reduced min^{-1} (mg protein)$^{-1}$.
[b]Specific activity of the washed membranes was 240nmol IDA min^{-1} (mg protein)$^{-1}$ determined in the oxygen consumption assay.
[c]Soybean phospholipids suspensions used for the reconstitution of IDA-DH were prepared in the presence of 1mM ubiquinone Q_{10}.

Table 4. Characterization of IDA-DH from *Chelatobacter heintzii* ATCC 29600 reconstituted in phospholipid vesicles.

Added compound (concn in mM)[a]	Relative activity (%)
Control[b]	5
Control + Ubiquinone Q_1 (1)	100
Control + Ubiquinone Q_0 (1)	22
Control + Duroquinone (1)	4
Control + Menadione (1)	7
Control + Phenazine methosulfate (1)	0
Control + Ubiquinone Q_1 (1) + Antimycine (0.5)	0
Control + Ubiquinone Q_1 (1) + DBMIB (0.5)	1

[a]The compounds indicated were present in the assay in addition to those present in the control experiment. The figure in brackets gives the final concentration (mM) of the added compound.
[b]The control assay contained in 1 ml of 50mM Tris-H_2SO_4 buffer (pH 8.0), 25mM $CaCl_2$, 42mM IDA, 2mM INT and reconstituted IDA-DH (168µg protein with a specific activity of 540nmol INT reduced min^{-1} (mg protein)$^{-1}$).

Table 5. Specific activities of NTA-MO, IDA-DH and Suc-DH in cells of *Chelatobacter heintzii* ATCC 29600 grown on different substrates.

Growth substrate	Specific activities [nmol substrate min^{-1} (mg protein)$^{-1}$] of:			
	NTA-MO	IDA-DH	Suc-DH	Sarcosine-DH
Succinate	<1	<1	39	<1
NTA	353	145	28	<1
IDA	226	52	38	<1
Dimethylglycine	<1	<1	97	60
Dimethylamine	<1	<1	28	n.d.

Neither NTA-MO activity nor IDA-DH activity was observed in extracts from cells that were grown on acetamide, choline, citrate, complex medium, glutamate, glycine, glycylglycine, glyoxylate, histidine, hypoxantine, methylamide, phenylalanine, proline, putrecine, thymine, trimethylamine, or threonine.
n.d. = Not determined.

type-b cytochromes (Crofts et al. 1983; Crofts & Wraight 1983). However, no inhibition of INT reduction was observed in the presence of 1 mM KCN, which inhibits electron transfer to oxygen at the level of the terminal oxidase.

Specific activities of IDA-DH and Suc-DH in membranes from Cb. heintzii *ATCC 29600.* To confirm the involvement of IDA-DH in the biodegradation of NTA to glycine and glyoxylate, *Cb. heintzii* ATCC 29600 was grown for five successive transfers (batch cultures) on succinate and ammonia followed by two transfers on the new substrate. From these cells, membrane fractions were tested for IDA-DH and Suc-DH activity and the cytosolic fractions were examined for NTA-MO activity (Table 5). Under all growth conditions membrane fractions contained Suc-DH activity, which was completely inhibited by 15 mM malonate. However, IDA-DH activity was only detected, when the bacteria were grown on either NTA or IDA. Similarly, NTA-MO was only expressed in cells grown on NTA or IDA. When the bacteria were grown on dimethylglycine or choline, membrane-bound activity of sarcosine dehydrogenase was observed. No membrane-bound N-methylglutamate dehydrogenase, as described by Bamforth & Large (1977) was present in membranes prepared from methylamine-, dimethylamine- or trimethylamine-grown cells.

Induction of IDA-DH in cells growing exponentially on succinate and ammonia. In order to investigate whether IDA-DH would be expressed in cells growing exponentially on a substrate other than IDA or NTA, *Cb. heintzii* ATCC 29600 was grown in batch culture with succinate and ammonia and 0.4 g NTA l^{-1} were pulsed to the exponentially growing culture (Fig. 2A). After a short lag succinate was utilized by the cells at a similar rate as before NTA addition. Four hours after the pulse a significant amount of NTA was already utilized and after 8 hours NTA was consumed to completion. IDA-DH (Fig. 2B) and the two components of the NTA-MO (Fig. 2C) were induced within the first hour after NTA addition, whereas the specific activity of Suc-DH was not influenced by pulsing NTA to the culture (Fig. 2B).

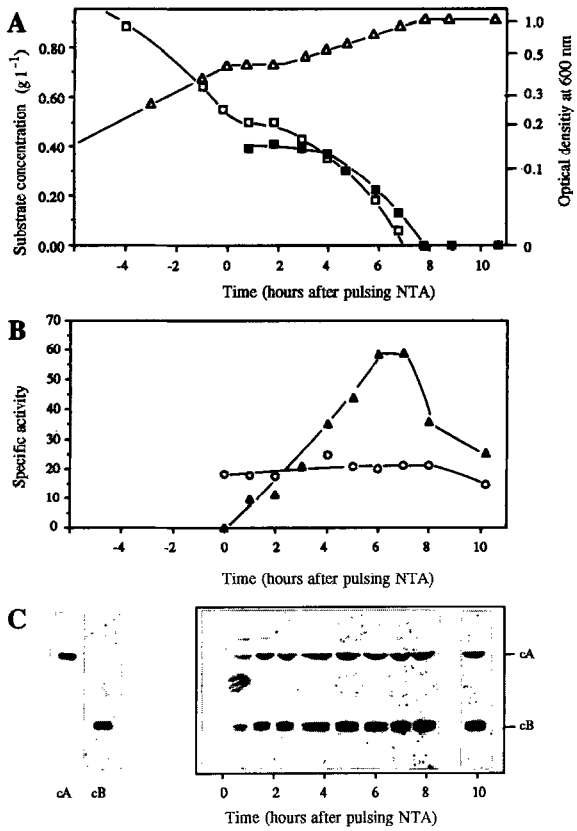

Fig. 2. Pulse of NTA to a culture of *Chelatobacter heintzii* ATCC 29600 growing exponentially in batch culture with succinate and ammonia.

A) Growth (OD_{600}) and concentration of substrates as a function of time. At time zero 0.4 g l^{-1} NTA was added to the culture medium. The symbols represent OD_{600} (△), the concentration of succinate (□) and NTA (■).

B) Specific activity of membrane-bound IDA-DH (▲) before and after NTA addition. The specific activity of Suc-DH (◇) was measured as a control. Specific activity is given in nmol substrate min^{-1} (mg protein)$^{-1}$.

C) Immunoblot of the two components cA and cB of NTA-MO. 34 µg protein from the cytosolic fraction were separated on SDS-PAGE and the two components of the NTA-MO were detected immunologically. 0.8 µg of purified cA and cB, respectively, from *Cb. heintzii* ATCC 29600 were included as standards.

Membrane-bound IDA-DH in other NTA-degrading bacteria. In order to investigate whether an inducible membrane-bound IDA-DH was also present in other NTA-degrading bacteria, membrane

fractions of nine bacterial strains from three different genera were examined and tested for IDA-DH and Suc-DH activity (Table 6). Both IDA-DH and Suc-DH activity were found in all strains tested. In membrane preparations from cells grown on glycine as a control experiment, Suc-DH activity (completely inhibited by 15 mM malonate) but no IDA-DH activity was found. In all strains tested IDA-DH was active in the presence of 15 mM malonate, but it was completely inhibited by 0.5 mM KCN. When inhibited by cyanide, membrane-bound IDA-DH from all strains catalyzed the rapid reduction of INT. Furthermore, NTA-MO activity was observed in the cytosolic fraction of eight out of nine strains when they were grown on NTA (Table 6). In the strain not exhibiting NTA-MO activity a PMS-dependent NTA dehydrogenase was found (Kemmler 1992).

Discussion

Two different enzymes are presently known to cleave NTA oxidatively, NTA monooxygenase and NTA dehydrogenase (Egli et al. 1990). Both enzymes have been purified and characterized extensively (Jenal-Wanner 1991; Kemmler 1992; Uetz et al. 1992) and for both enzymes the products formed from NTA were IDA and glyoxylate. In contrast to earlier reports which suggested that NTA-MO might be involved in the subsequent oxidation of IDA (Firestone & Tiedje 1978), neither enzyme was reported to exhibit activity with IDA.

The data reported here demonstrate that in all the presently known Gram-negative NTA-degrading bacteria IDA is metabolized by a membrane-bound IDA-DH. This enzyme is of considerable importance in the biodegradation of NTA because accumulation of IDA in the natural environment would be unfavorable because of the possible formation of putatively cancerogenic N-nitroso-IDA from IDA and nitrite (Epstein 1972; Pickaver 1976).

As with NTA-MO and NTA dehydrogenase, the substrate specificity of IDA-DH appears to be very narrow. Presently IDA is the only known substrate and IDA-DH exhibited no significant activity with several structurally similar compounds, such as succinate, glutarate, N-methylglutamate, N-methyl-IDA and sarcosine. Nevertheless, the high K_m value (8 mM) observed for Ca^{2+} complexed IDA suggests that either the protein has evolved from an already existing protein or that IDA is not the natural substrate for this enzyme. From the structural resemblence of the substrates both sarcosine dehydroge-

Table 6. Occurence of IDA-DH and NTA-MO in nine different NTA-degrading strains.

Genus	Strain	Specific activities [a] [nmol substrate min^{-1} (mg protein)$^{-1}$]		
		IDA-DH	Suc-DH	NTA-MO
Chelatococcus asaccharovorans	TE 2	32	30	26
Chelatobacter heintzii	ATCC 29600	25	22	148
	TE 4	7.5	7.5	168
	TE 5	15	9.4	152
	TE 6	28	10	118
	TE 7	23	6.3	152
	TE 8	8.5	6.5	152
	TE 10	40	14	120
Unidentified genus[c]	TE 11	10	13.3	<5[b]

[a] Specific activity of IDA-DH was determined using the oxygen consumption assay in the presence of 20 mM $CaCl_2$.

[b] A PMS-dependant NTA dehydrogenase, as reported by Kemmler (Kemmler 1992), was present in the cytosolic fraction.

[c] Strain TE 11 was characterized by (Wanner et al. 1990). This strain probably belongs to a new genus within the γ-subgroup the *Proteobacteria.*

nase and Suc-DH are two potential candidates. However, in addition to the fact that neither enzyme accepted IDA as a substrate (Table 5) and that they were not induced either by NTA or by IDA, they also differ structurally from IDA-DH. By applying the method described by Reddy & Weber (1986), it was found that IDA-DH, sarcosine dehydrogenase and Suc-DH differ in their mobility on native polyacrylamide gels (T. Uetz & T. Egli unpubl.).

The fact that IDA-DH was present in the particulate fraction of cell-free extracts, that its activity was linked to the respiratory chain and that IDA-DH activity could only be solubilized by using detergents, demonstrates that it forms an integral protein complex in the membrane. From this one can conclude that IDA-DH is clearly distinct from other soluble dehydrogenases such as N-methylglutamate dehydrogenase (Bamforth & Large 1977; Boulton et al. 1980), dimethylamine dehydrogenase, trimethylamine dehydrogenase (Kasprzak et al. 1983; Meiberg & Harder 1979), and from dehydrogenases that are only loosely associated with the cytoplasmic membrane, such as sarcosine dehydrogenase, choline dehydrogenase and D-alanine dehydrogenase from *Pseudomonas aeruginosa* (Bater & Venables 1977). Many membrane-bound dehydrogenases have been described in the literature which have ubiquinones as natural electron acceptors. However, in contrast to IDA-DH, all these dehydrogenases were able to reduce PMS after solubilization with detergents and activity was not reduced after solubilization. For the majority of those dehydrogenases partial or complete purification was therefore possible (Ingledew & Poole 1984; Matsushita et al. 1987; Olsiewski et al. 1980; Pennoyer et al. 1988; Tushurashavili et al. 1985). Others consisted of large complexes and purification turned out to be difficult, as it was reported for succinate dehydrogenase from *E. coli* (Kita et al. 1989) or N-methylglutamate dehydrogenase from *Pseudomonas aminovorans* (Bamforth & Large 1977). The latter two enzymes contained b-type cytochromes. This also seems to be the case for IDA-DH, because IDA-DH reconstituted in soybean phospholipids was sensitive to antimycin A. Further indication for the presence of a b-type cytochrome in IDA-DH was obtained from preliminary difference spectra, where an IDA-reduced reconstituted solubilized IDA-DH versus an oxidized control exhibited a maximum at 560nm with a shoulder at approximately 580nm (T. Uetz & T. Egli unpubl.). This, however, remains to be confirmed by partial or complete purification of IDA-DH.

An interesting aspect of NTA degradation by *Cb. heintzii* ATCC 29600 is its regulation. Although this bacterium is able to grow on a large variety of substrates as a sole source of carbon, nitrogen and energy (Egli et al. 1988) none of the various substrates tested other than NTA or IDA led to the induction of either NTA-MO or IDA-DH, not even when the substrates were structurally closely related to NTA and IDA. The fact that the two enzymes were induced within one hour after NTA was pulsed to a culture growing exponentially on succinate and ammonia, suggests that NTA and IDA (or metabolites thereof) function as inducer molecules, which leds to *de novo* synthesis of NTA-MO and probably also of IDA-DH. Nevertheless, with respect to the regulation of enzymes involved in the metabolism of NTA several ecological aspects remain to be investigated. For example, it would be interesting to know whether the NTA-degrading enzymes are also expressed under environmentally relevant conditions, e.g. during growth at low substrate concentrations with mixtures of carbon/nitrogen sources including NTA or IDA. This challenging question concerning inducibility of NTA-MO and IDA-DH in ecosystems could be investigated using the membrane diffusion chamber technique as described by McFeters et al. (1990), where pure cultures of bacteria can be exposed to river water or wastewater. Preliminary experiments have already shown that *Cb. heintzii* ATCC 29600, pre-grown in the laboratory under non-inducing conditions, was able to catabolize NTA within 6–8 hours upon transfer in a diffusion chamber to a wastewater treatment plant.

A striking result was that all the bacterial strains studied, belonging to three different genera contain a membrane-associated IDA-DH. This underlines the important role of this enzyme in the biodegradation of NTA. The involvement of IDA-DH in the biodegradation of other anthropogenic compounds is also possible, e.g. IDA was reported to be an in-

termediate in the biodegradation of EDTA (Belly et al. 1975). Furthermore, recently an enzyme able to oxidize the herbicide glyphosate ($HOOC\text{-}CH_2\text{-}NH\text{-}CH_2\text{-}PO_3H_2$), which had a higher affinity for IDA than for glyphosate, was cloned from a glyphosate-utilizing *Pseudomonas* strain (R.E. Dick pers. comm.). IDA-DH from *Cb. heintzii* ATCC 29600 had no affinity for glyphosate, because this compound was neither a substrate nor an inhibitor for our enzyme preparation (T. Uetz & T. Egli unpubl.).

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