

Purification and Characterization of a Two-Component Monooxygenase That Hydroxylates Nitrilotriacetate from “*Chelatobacter*” Strain ATCC 29600

THOMAS UETZ,^{1,2} RENÉ SCHNEIDER,^{1†} MARIO SNOZZI,¹ AND THOMAS EGLI^{1*}

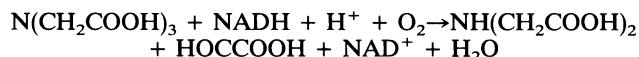
Swiss Federal Institute for Water Resources and Water Pollution Control, Swiss Federal Institutes of Technology, CH-8600 Dübendorf,¹ and Department of Microbiology, Biozentrum, University of Basel, CH-4056 Basel,² Switzerland

Received 27 August 1991/Accepted 10 December 1991

An assay based on the consumption of nitrilotriacetate (NTA) was developed to measure the activity of NTA monooxygenase (NTA-Mo) in cell extracts of “*Chelatobacter*” strain ATCC 29600 and to purify a functional, NTA-hydroxylating enzyme complex. The complex consisted of two components that easily dissociated during purification and upon dilution. Both components were purified to more than 95% homogeneity, and it was possible to reconstitute the functional, NTA-hydroxylating enzyme complex from pure component A (cA) and component B (cB). cB exhibited NTA-stimulated NADH oxidation but was unable to hydroxylate NTA. It had a native molecular mass of 88 kDa and contained flavin mononucleotide (FMN). cA had a native molecular mass of 99 kDa. No catalytic activity has yet been shown for cA alone. Under unfavorable conditions, NADH oxidation was partly or completely uncoupled from hydroxylation, resulting in the formation of H₂O₂. Optimum hydroxylating activity was found to be dependent on the molar ratio of the two components, the absolute concentration of the enzyme complex, and the presence of FMN. Uncoupling of the reaction was favored in the presence of high salt concentrations and in the presence of flavin adenine dinucleotide. The NTA-Mo complex was sensitive to sulfhydryl reagents, but inhibition was reversible by addition of excess dithiothreitol. The *K_m* values for Mg²⁺-NTA, FMN, and NADH were determined as 0.5 mM, 1.3 μM, and 0.35 mM, respectively. Of 26 tested compounds, NTA was the only substrate for NTA-Mo.

The complexing agent nitrilotriacetate (NTA) is used for a range of different purposes, and one of its most controversial applications is that as a substitute for sodium triphosphate in laundry detergents (28). Many representatives of both obligately aerobic and facultatively denitrifying microorganisms which can use NTA as a sole source of nitrogen, carbon and energy have been isolated. The majority of such isolates are gram-negative, obligately aerobic rods (1, 5, 10, 14, 29) which previously have been identified as *Pseudomonas* spp. Recently, it has been shown that these isolates belong to a new genus for which the name “*Chelatobacter*” has been proposed (6).

The biochemical pathway for NTA degradation was first investigated in the two virtually identical “*Chelatobacter*” isolates T23 (1) and ATCC 29600 (9). In both strains, a monooxygenase was reported to be responsible for the oxidative conversion of NTA to iminodiacetate (IDA) and glyoxylate, as follows:



Firestone and Tiedje proposed that NTA was hydroxylated at the α carbon of the molecule, with the subsequent spontaneous formation of IDA and glyoxylate (8). Attempts to purify NTA monooxygenase (NTA-Mo) by using an enzyme assay based on the spectrophotometric determination of NTA-dependent NADH oxidation resulted in the isolation of a protein which was unable to hydroxylate NTA (8, 26). This suggested that NTA-Mo was composed of at least two components and that purification had to be based

on measuring activity via the consumption of NTA. Therefore, methods were developed to analyze NTA and IDA in cell extracts (24, 25). In this paper, the characterization of the NTA-Mo in cell extracts, as well as the purification, reconstitution, and characterization of a functional two-component NTA-Mo, is reported.

MATERIALS AND METHODS

Growth of the microorganism. “*Chelatobacter*” strain ATCC 29600 was obtained from the American Type Culture Collection, Rockville, Md., and was maintained on a synthetic medium containing 1 g of NTA liter⁻¹ as described previously (5). In order to avoid excretion of large amounts of ammonia, the strain was grown on a mixture of NTA and acetate (1 g of each liter⁻¹) for large-scale growth (100 liters). Towards the end of the exponential phase, additional NTA and acetate (1 g of each liter⁻¹) was given to the culture and the pH was kept in the range of 6.5 to 7.5 by discontinuous addition of 0.5 M *ortho*-phosphoric acid. The 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.

Determination of NTA-Mo activity by four assays. Four different assays were developed to measure the activity of NTA-Mo.

(i) **NTA-dependent NADH oxidation.** Assays were conducted in 30 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.8 (pH optimum in cell extracts), at 25°C. Buffer (0.7 ml) containing 10 μM flavin mononucleotide (FMN), 2 mM MgCl₂, 0.2 mM NADH, and a sample of unknown component B (cB) content (not exceeding 0.1 U) was placed in a 1-cm quartz cuvette. The reaction was started by the addition of 2 mM NTA, and NADH oxidation was monitored spectrophotometrically at

* Corresponding author.

† Present address: School for Microbiology, University of New South Wales, Kensington, Australia.

340 nm in a Uvicon 860 spectrophotometer (Kontron, Switzerland). Nonspecific NADH oxidation was subtracted to obtain the NTA-dependent NADH oxidation, and the activity, expressed as micromoles minute⁻¹ (1 U), was calculated by using the molar extinction coefficient for NADH, ϵ , equivalent to $6,200 A_{340} \text{ cm}^{-1} \text{ mol}^{-1}$.

(ii) **Glyoxylate formation.** This qualitative assay was used for rapid estimation of the component A (cA) content in fractions from chromatography columns. The assay is based on the end-point measurement of glyoxylate formed after 10 min of reaction time. Fractions from chromatography columns to be measured were desalted by filtration through a Pharmacia PD 10 Sephadex G-25 column, and 60 μl of the desalted sample (protein concentration in the range of 1 to 5 mg ml⁻¹) was placed in a plastic tube together with 10 μl of cB (approximately 0.05 U), 2 mM NTA, 2 mM MgCl₂ and 10 μM FMN in a total volume of 100 μl of 20 mM HEPES, pH 7.8. In addition, 5 μg of catalase was added to the assay mixture to avoid accumulation of H₂O₂ during the reaction, because in preliminary experiments it was found that the H₂O₂ reacts with glyoxylate (23). The reaction was started by the addition of 5 mM NADH, and the tube was shaken continuously for 10 min. The reaction was stopped by the addition of 40 μl of 25 mM HCl, and samples were analyzed for glyoxylate as described below.

(iii) **NTA consumption.** The assay was used to characterize the reconstituted enzyme. The assay was conducted in a glass vial, with a diameter of 18 mm, containing a star-shaped magnetic stirring bar, with a volume of 0.5 ml of 40 mM TAPS [tris(hydroxymethyl)methylaminopropane sulfonic acid] buffer at pH 8.5, because optimum activity of the reconstituted NTA-Mo was observed at this pH. The buffer contained 10 μM FMN, 10 μg of catalase ml⁻¹, 2 mM MgCl₂, 2 mM NTA, and enzyme. The reaction was started by the addition of NADH to a final concentration of 3.4 mM. The assay was carried out with vigorous stirring at 25°C. Samples of 100 μl were removed from the assay at intervals of 2 min; they were acidified to stop the reaction and were analyzed for NTA, IDA, or glyoxylate as described below.

(iv) **Oxygen consumption.** This assay was used to characterize the reconstituted NTA-Mo and the rate of uncoupling of NADH oxidation from NTA hydroxylation. Activity of NTA-Mo was determined in a closed reaction vessel (0.8-ml total volume) fitted with a Clarke-type oxygen electrode and a magnetic stirrer (Yellow Springs Instruments, Yellow Springs, Ohio). The electrode was calibrated by adding defined amounts of H₂O₂ (0 to 0.8 μmol) to N₂-saturated buffer and recording the increase of oxygen after the addition of 10 μg of catalase.

For the assays, cA and cB, 2 mM NTA, 2 mM MgCl₂, 10 μM FMN, and 40 mM TAPS buffer (pH 8.5) were placed in the reaction vessel. The reaction was started by the addition of 200 nmol of NADH, and the decrease of O₂ concentration was monitored.

Immunoquantification of the two NTA-Mo components. Protein samples were run on sodium dodecyl sulfate (SDS)-12% polyacrylamide gels and blotted onto a nitrocellulose sheet, as described by Towbin et al. (30). The nitrocellulose sheet was incubated in 20 ml of phosphate-buffered saline (PBS) (100 mM KNaPO₄ [pH 7.2]-150 mM NaCl) containing 4% PVP (polyvinylpyrrolidone) for 2 h. Subsequently, it was incubated in 20 ml of PBS containing 2% PVP, 10 μl of antiserum against cA, and 10 μl of antiserum against cB for 1 h. This was followed by extensive washing with PBS containing 2% PVP and incubation in PBS containing 2% PVP and 1 μCi of ¹²⁵I-labeled protein A for 45 min (13). After

the sheet was washed with PBS and dried, it was exposed for 60 h to a Fuji RX medical X-ray film. For quantification of cA and cB, the developed film was scanned with a computing densitometer model 300A gel scanner (Molecular Dynamics, Sunnyvale, Calif.).

Analysis of NTA. To measure NTA in cell extracts and fractions from protein purification, trichloroacetic acid was added to samples to a final concentration of 5% (vol/vol) and precipitated proteins were removed by centrifugation for 5 min at 10,000 $\times g$. NTA was analyzed by high-pressure ion-exclusion chromatography, as described by Schneider et al. (25).

Analysis of IDA. A 100- μl sample was mixed with 200 μl of 30 mM H₃PO₄ and boiled for 5 min, and the precipitated protein was removed by centrifugation. A total of 215 μl of the supernatant was mixed with 500 μl of H₂O before IDA was determined by a modified procedure of Schneider et al. (24). Separation was achieved on a Dionex AS4A column using 2 mM tyrosine in 4.5 mM NaOH as the eluent with a Dionex 2000i high-pressure chromatography system connected to a CDM2 conductivity detector (Dionex, Sunnyvale, Calif.).

Analysis of glyoxylate. Prior to analysis of glyoxylate, samples of 100 μl from the NTA consumption assay were mixed with 40 μl of 25 mM HCl to stop the NTA-Mo reaction. The amount of glyoxylate in the sample was analyzed with the phenylhydrazine-K₃Fe(CN)₆ method described by Trijebels and Vogels (31) but with a total assay volume of 280 μl .

Determination of H₂O₂. The amount of H₂O₂ formed in the oxygen reaction vessel was determined from the amount of oxygen formed after the addition of 10 μg of catalase ml⁻¹ to the reaction vessel.

Protein determination. Protein concentrations were measured by using the bicinchoninic acid protein assay (Pierce, Rockford, Ill.). Bovine serum albumin was used as the standard.

Purification of NTA-Mo. If not otherwise stated, all manipulations were carried out at 4°C and all buffers contained 2 mM dithiothreitol (DTT) and 0.2 mM phenylmethylsulfonyl fluoride (PMSF) as a proteinase inhibitor (15). The pH of all buffers was adjusted at the temperature at which they were used.

(i) **Preparation of cell extract.** A total of 60 g (wet weight) of frozen cell paste of "*Chelatobacter*" strain ATCC 29600 was suspended in 400 ml Tris-HCl buffer (pH 8.0) containing 0.5 mM PMSF, 10 mg of DNase I, 5 mM MgSO₄, and the protease inhibitors leupetin and pepstatin (15) at a concentration of 1 μM . After homogenization for 1 min in a Sorvall homogenizer, the cells were broken by a single passage through a French press (Aminco, Urbana, Ill.) at 15,000 lb/in². EDTA was added to a final concentration of 10 mM, and the suspension was centrifuged for 30 min at 40,000 $\times g$ to remove unbroken cells and cell debris.

(ii) **Separation of the two components.** Cell extract was centrifuged for 2 h at 160,000 $\times g$. To the supernatant (440 ml), 18 mg of PMSF was added, together with ammonium sulfate to 33% saturation. After being stirred for 15 min, the extract was centrifuged for 10 min at 10,000 $\times g$. The pellet contained no activity and was discarded. The supernatant was chromatographed on a phenyl-Sepharose CL-4B column (130 by 50 mm; Pharmacia, Uppsala, Sweden) equilibrated with 40 mM Tris-HCl, pH 8.0, containing 25% saturated ammonium sulfate and 1 mM EDTA. After being loaded, the column was first washed with 500 ml of equilibration buffer before the proteins were eluted with a 1.5-liter linear simul-

taneous gradient of ammonium sulfate from 25 to 0% saturation and of ethylene glycol from 0 to 20% (vol/vol) at a flow rate of 400 ml/h.

(iii) **Seventy-five percent ammonium sulfate precipitation.** First, cB was purified to homogeneity before fractions from the phenyl-Sepharose could be monitored for cA content. Pooled fractions from the phenyl-Sepharose column containing cA or cB were brought to approximately 75% ammonium sulfate. After being stirred for 15 min, the precipitated proteins were collected by centrifugation (10 min at $8,000 \times g$) and resuspended in 30 ml of 40 mM Tris-HCl (pH 8.0) buffer. The two protein solutions (containing either cA or cB) were then treated individually as described below.

(iv) **Chromatography on TMAE-Fractogel column.** Protein solutions were desalted by being passed through a Pharmacia PD 10 Sephadex G-25 column before being chromatographed on a trimethylaminoethyl (TMAE) anion-exchange column (Fractogel EMD TMAE-650, 10 by 150 mm; Merck, Darmstadt, Germany) with a fast protein liquid chromatography (FPLC) system (Pharmacia). The column was equilibrated with 40 mM Tris-HCl (pH 8.0), and the proteins were eluted with a 150-ml linear NaCl gradient from 0 to 300 mM.

(v) **Chromatography on phenyl-Superose column.** Protein solutions were first brought to 20% saturation with ammonium sulfate and filtered through a 0.45- μ m-pore-size Millipore Millex-HA filter before being loaded at a flow rate of 1 ml/min on a Pharmacia phenyl-Superose HR 10/10 column (10 by 100 mm) by using FPLC. The column was equilibrated with 0.1 M K_2HPO_4 - KH_2PO_4 buffer at pH 7.5 saturated to 20% with ammonium sulfate, and the proteins were eluted with a 100-ml linear gradient from 20 to 0% ammonium sulfate saturation.

Preparation of antisera. Purified cA or cB (250 μ g) in 0.5 ml of PBS was mixed with an equal volume of complete Freund's adjuvant and was injected subcutaneously into each of the two rabbits. A secondary injection was done after 4 weeks with 250 μ g of the proteins but mixed with Freund's incomplete adjuvant. Blood was collected 2 weeks after the second injection, and polyclonal antisera were produced according to general procedures previously described (13). Specificities of the two antisera were tested by the immunoblotting technique described above. None of the sera reacted against proteins of cell extracts from succinate-grown "*Chelatobacter*" strain ATCC 29600, nor did the antibodies against cA react with cB and vice versa (33).

SDS-PAGE. Discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) was performed following the protocol of Laemmli (16), with (13 by 90 by 1.5 mm) 12.5% polyacrylamide slab gels. The following standard proteins for subunit-molecular-mass determination were obtained from Pharmacia: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α -lactalbumin (14.4 kDa). Gels were stained with Coomassie brilliant blue R-250, or proteins were blotted to nitrocellulose by the procedure of Towbin et al. (30).

Analytical ultracentrifugation. Sedimentation velocity and sedimentation equilibrium runs were performed on a Spinco model E analytical ultracentrifuge equipped with absorption optics and a photoelectric scanning system (Beckman, Palo Alto, Calif.). Sedimentation velocity and equilibration runs were carried out in an AN-D rotor at 56,000 rpm and 21°C and 12,000 rpm and 18°C, respectively, by using a 12-mm double-sector Epon cell. For sedimentation equilibrium runs, only 0.11 ml of solution and solvent was filled in each sector. The protein concentrations used were 0.3 mg ml⁻¹

for cA and 0.6 mg ml⁻¹ for cB (in PBS). Scanner tracings during runs were taken at 280 nm and, for cB, also at 450 nm in the sedimentation velocity run. The molecular masses and the sedimentation coefficients were determined according to the method of Schachman (22), with an estimated value of 0.73 ml g⁻¹ for the partial specific volume. Buffer density as well as buffer viscosity was taken into account.

Chromatography of flavins. A solution of 0.6 mg of cB ml⁻¹ in PBS was treated with 5% trichloroacetic acid and centrifuged for 2 min in an Eppendorf centrifuge at 14,000 rpm. The supernatant was analyzed by high-pressure liquid chromatography (Millipore-Waters, Milford, Mass.) on a Waters μ Bondapak C18 column with the following elution buffer: 0.1 M ammonium acetate (pH 2.7), 10% (vol/vol) acetonitrile, and 1% (vol/vol) methanol. The flow rate was 0.5 ml min⁻¹, and the A_{260} on a scale of 0 to 0.02 U was recorded. The retention times of flavin adenine dinucleotide (FAD), FMN, and the flavin component extracted from cB were 14.70, 16.20, and 16.65 min, respectively.

Iron content of the two NTA-Mo components. The iron content of solutions of the enzyme in 10 mM HEPES buffer (pH 7.5) was measured with a Thermo Charrel Ash Video E12 atom absorption spectrometer. The instrument was calibrated with solutions of $NH_4Fe(SO_4)_2$ containing 10, 20, 40, and 80 μ M of Fe. The buffer without enzyme was used for measuring the background iron concentration.

Chemicals. Catalase, DTT, DNase I, and PMSF were obtained from Serva, Heidelberg, Germany. Leupeptin and pepstatin were products of Boehringer, Mannheim, Germany. *N*-methylated amino acids, *p*-hydroxymercuribenzoate, and 5,5'-dithio-bis-(2-nitrobenzoate) were purchased from Sigma, St. Louis, Mo. All other chemicals were of analytical grade and were obtained from either Fluka, Buchs, Switzerland, or Merck.

RESULTS

Characterization of NTA-Mo in cell extract. Measurement of NTA-Mo activity was complicated by the fact that the spectrophotometric assay does not distinguish between the uncoupled oxidation of NADH and the hydroxylation of NTA. Therefore, an NTA consumption assay was developed on the basis of the determination of either the consumption of NTA or the production of IDA by using high-pressure ion chromatography (24, 25). The stoichiometry of the NTA-Mo reaction was determined in cell extracts (Fig. 1). The enzyme exhibited a linear reaction rate over a 20-min period, and the stoichiometric formation of IDA and glyoxylate from NTA was observed. A total of 1.5 mol of NADH per mol of NTA consumed was oxidized, and the reaction was strictly dependent on the addition of Mg^{2+} or Co^{2+} ions. Mn^{2+} ions were able to replace Mg^{2+} but led to a higher uncoupled NADH oxidation (2.6 mol of NADH per mol of NTA consumed). No NTA consumption was observed with Ca^{2+} , Fe^{2+} , Fe^{3+} , Zn^{2+} , Cu^{2+} , or Ni^{2+} . NTA-Mo activity increased by a factor of 4 when 3 μ M FMN was included in the assay mixture. Both pH and temperature dependence of NTA-Mo activity were determined in cell extracts. The pH optimum for NTA consumption in cell extracts was 7.8. At pH 7.2 and 8.4, 25% of the maximum activity was obtained, and no activity was left at pH 6 or 9. The optimum temperature for NTA-Mo was 25°C, 12% activity was left at 2°C, and no activity was observed at 40°C. No significant difference was observed when either Co^{2+} or Mn^{2+} instead of Mg^{2+} was used in the assay. In cell extract, NTA-Mo activity was rather unstable. When stored at 4°C in 50 mM

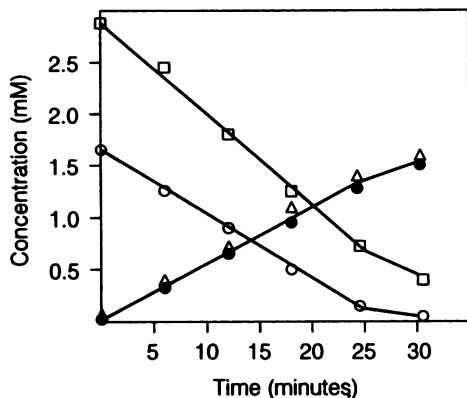


FIG. 1. Stoichiometry of the reaction catalyzed by NTA-Mo in cell extracts. The reaction was performed in the presence of 1.6 mM MgCl_2 , 9 μM FMN, and 230 μg of protein ml^{-1} in 30 mM HEPES buffer (pH 7.8) at 30°C. The symbols represent concentrations of NADH (□), NTA (○), IDA (●), and glyoxylate (△).

Tris-HCl, 50% of the initial activity was lost within 200 h when both protease inhibitors and DTT were excluded.

Strategy for the purification of NTA-Mo. The major problem encountered during the purification of NTA-Mo was the loss of NTA consumption activity. With the spectrophotometric assay, it was possible to purify a protein that exhibited NTA-stimulated NADH oxidation but was completely devoid of NTA-consuming activity. This suggested that NTA-Mo was composed of at least two components: one component exhibiting NTA-stimulated NADH oxidation with the concomitant formation of H_2O_2 and a second component which enabled hydroxylation of NTA in the presence of the former component and NADH (Fig. 2). There were two possible strategies to purify functional NTA-Mo: (i) purification of the enzyme as an active complex or (ii) separation of the two components, purification of the individual components, and subsequent reconstitution of the active enzyme complex. Various attempts (gel filtration on Sephadex G-100 and chromatography on a phenyl-Sepha-

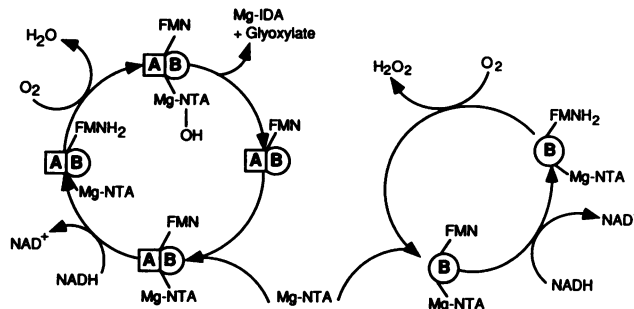


FIG. 2. Proposed scheme for the reactions catalyzed by NTA-Mo. The right-hand circle illustrates the reaction mediated by pure cB, whereas the left-hand circle shows the situation for the complete NTA-Mo comprising both components. A, cA; B, cB.

rose column or DEAE-Sepharose column) to purify the whole enzyme complex failed because most of the NTA-consuming activity was lost (23). Since preliminary experiments showed that reconstitution of activity was possible, the second strategy was adopted. For this, cB was first purified to homogeneity following NTA-dependent NADH oxidation in the spectrophotometer. Subsequently, cB was used to purify cA by reconstituting the active enzyme complex and measuring activity qualitatively via NTA consumption. Later, purification of cA was achieved also by employing the immunoquantification assay.

Purification of cB. The results of the purification of cB are shown in Table 1. The two components were separated by chromatography on phenyl-Sepharose, in which cB eluted after cA. Pooled fractions exhibiting NTA-stimulated NADH oxidation were concentrated by ammonium sulfate precipitation, desalted, and further purified on a TMAE anion-exchange column. Active fractions, which eluted at concentrations between 200 and 250 mM NaCl, were pooled and subjected to chromatography on a phenyl-Superose column. Active fractions eluted between 4 and 1% ammonium sulfate saturation. Small aliquots of cB were frozen in liquid nitrogen and stored at -70°C without significant loss of activity over several months.

TABLE 1. Purification of cA and cB of NTA monooxygenase from "*Chelatobacter*" strain ATCC 29600

Component and purification step (lane number in Fig. 3)	Vol (ml)	Total protein (mg)	cA (mg) ^a	cB (mg) ^a	Purification factor ^b	% Recovery	cB activity (U)
cA							
Cell extract (1)	450	3,645	150	88	1	100	
Ultracentrifugation (2)	440	2,860	166	89	1.4	110	
33% Ammonium sulfate (3)	490	1,740	160	76	2.3	106	
Phenyl-Sepharose (11)	265	556	97	36	4.3	65	
75% Ammonium sulfate (12)	35	277	58	17	5.2	38	
TMAE-Fractogel (14)	48	91	68	0	18.1	45	
Phenyl-Superose (15)	24	60	57	0	23.1	38	
cB							
Cell extract (1)	450	3,645	150	88	1		1,316
Ultracentrifugation (2)	440	2,860	166	89	1.2		1,214
33% Ammonium sulfate (3)	490	1,740	160	76	3		1,907
Phenyl-Sepharose (4)	205	308	17	59	10		1,139
75% Ammonium sulfate (5)	28	201	23	27	9		669
TMAE-Fractogel (7)	26	31	0	19	47		546
Phenyl-Superose (8)	9	13	0	12	111		514

^a Estimated by immunoquantification.

^b For cA, the purification factor was calculated from the results of the immunoquantification; for cB, the purification factor was estimated from the remaining NADH oxidation activity determined at the end of the purification.

Purification of cA. After separation of the two components, fractions containing cA were pooled, concentrated by ammonium sulfate precipitation, desalted, and further purified on a TMAE anion-exchange column. Active fractions, which eluted at concentrations between 140 and 200 mM NaCl, were pooled and further chromatographed on phenyl-Superose. Fractions containing cA, eluting between 9 and 6% ammonium sulfate saturation, were pooled, frozen in liquid nitrogen, and stored at -70°C . cA was stable over a 2-week period at 4°C , even in the absence of DTT. The largest loss of cA occurred during incomplete separation of the two components on phenyl-Sepharose. A recovery of 38% and a 23-fold purification were finally achieved (Table 1). The Coomassie blue-stained SDS-polyacrylamide gel of pooled fractions from individual purification steps and the corresponding immunostained Western blot are shown in Fig. 3. Both components were judged to be more than 95% homogeneous, and neither of the two components was contaminated with the other.

Stability of cB. The stability of purified cB was tested in the presence of several stabilizing agents. A rapid loss of NTA-dependent NADH oxidation activity was observed at 4°C in 20 mM HEPES buffer at pH 7.8 (60% loss within 7 days). Addition of 2 mM DTT stabilized the activity significantly (20% loss within 7 days), and combined addition of 2 mM DTT and ammonium sulfate to 5% saturation increased the stability further (5% loss within 7 days). This is consistent with the fact that the largest loss of activity during purification was observed when the collected fractions from the phenyl-Sepharose column had to be concentrated by ammonium sulfate precipitation and desalted to be loaded on the anion-exchange column (Table 1). The use of immunquantification assay to monitor the purification procedure revealed that an extensive loss not only of activity but also of the original cB protein occurred at this purification step (Table 1 and Fig. 3B). In Fig. 3B, a protein fragment still cross-reacting with cB antiserum appeared on the Western blot of SDS-PAGE-separated proteins, suggesting that an inactive form of cB was formed. At -70°C in the presence of 2 mM DTT, purified cB was stable over several months.

Molecular masses of the two components. The molecular masses of the monomers of cA and cB were calculated from comparison of their mobilities on SDS-polyacrylamide gels and were found to be 47 and 36 kDa, respectively (Fig. 3; Table 2). From sedimentation equilibrium experiments, the molecular masses of the native components were determined as 99 and 88 kDa, respectively (Table 2). The sedimentation velocity coefficient of cA was 5.95 and that of cB was 4.6 (Table 2). This confirmed the homogeneity of the two components and suggested a globular shape. Thus, both cA and cB most probably occur as dimers.

Cofactors of the two components. cB has a typical flavin spectrum with absorption maxima at 278, 375, and 457 nm (spectrum not shown). The nature of the flavin in cB was determined by high-pressure liquid chromatography and was identified as FMN. At the maximum, 0.4 mol of FMN per mol of purified cB was bound; this amount is consistent with the fact that NTA-dependent NADH oxidation was stimulated severalfold by the addition of FMN. Further evidence for the loss of FMN during purification comes from the fact that it was possible to resaturate cB containing less than 0.1 mol of FMN per mol of protein by exposure to phosphate buffer containing 40 μM FMN and 5 mM DTT for 84 h. Bound flavin could be fully reduced by the addition of excess sodium dithionite or NADH, resulting in the disappearance of the absorption maximum at 457 nm. Resaturated cB

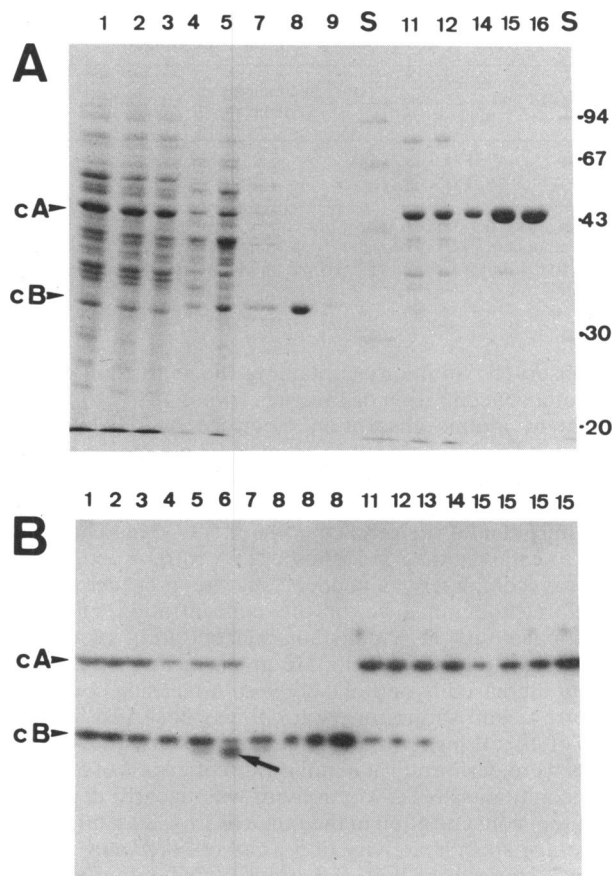


FIG. 3. SDS-PAGE of the two components of NTA-Mo at different stages during the purification. (A) Coomassie blue-stained gel; (B) the corresponding autoradiography of the immunostained Western blot. Lanes (numbers are cross-referenced to the purification steps listed in Table 1): 1, cell extract; 2, cytosolic fraction; 3, 33% ammonium sulfate cut; 4, pooled fractions of cB from phenyl-Sepharose; 5, 75% ammonium sulfate cut from the purification of cB; 6, desalted 75% ammonium sulfate cut from the purification of cB; 7, pooled fractions of cB from TMAE-Fractogel; 8, pooled fractions of cB from phenyl-Superose (0.4, 0.9, and 1.7 μg of cB); 9, pooled fractions of cB from Superose; 11, pooled fractions of cA from phenyl-Sepharose; 12, 75% ammonium sulfate cut from the purification of cA; 13, desalted 75% ammonium sulfate cut from the purification of cA; 14, pooled fractions of cA from TMAE-Fractogel; 15, pooled fractions of cA from phenyl-Superose (0.2, 0.4, 1.2, and 2.4 μg of cA); 16, pooled fractions of cA from Superose. The arrow (in panel B) indicates the product formed as a result of the decay of cB. When assessed with immunquantification at 2, 48, and 96 h after desalting, this fragment increased in quantity with time, whereas the quantity of cB decreased correspondingly. In panel A, molecular mass markers (in kilodaltons) are shown on the right.

contained 0.97 mol of FMN per mol of native cB (88 kDa). This was calculated from the decrease in A_{455} upon reduction, by using an extinction coefficient for FMN at 455 nm of 12,500. In all subsequent experiments, cB containing approximately 0.1 mol of FMN per mol of native cB was used. No flavin was found in cA even after 84 h of exposure to excess FMN.

Iron content of the two components. When the components were exposed to 10 mM EDTA for 20 min, passed through a Pharmacia PD 10 desalting column, and equilibrated with EDTA-free HEPES (pH 7.6), no loss of NTA-Mo activity

TABLE 2. Properties of the two components of the NTA-Mo from "*Chelatobacter*" strain ATCC 29600

Component	Mol mass (Da)		Sedimentation coefficient	Fe content ^a	FMN present ^b
	SDS	Native			
cA	47,000	99,000	5.95	<0.15	No
cB	36,000	88,000	4.50	<0.15	Yes

^a Moles of Fe per mole of native protein.

^b The amount of FMN in cB varied, depending on the preparation, from less than 0.1 to 0.4, but was 1 mol of FMN per mol of native cB after resaturation with FMN.

was observed. Solutions containing the individual components at a concentration of 1 mg ml⁻¹ were analyzed for iron content by atomic absorption spectroscopy. Both components contained less than 0.15 atom of Fe per mol of protein, indicating that neither Fe-sulfur clusters nor cytochromes are present in cA or cB.

Optimization of the reconstitution of NTA-Mo. The ability of cB to catalyze the oxidation of NTA to IDA and glyoxylate was completely dependent on the presence of cA. Therefore, the influence of the concentration of cA on catalytic activity at a constant concentration of cB was investigated, and the results are given in Fig. 4. Catalytic activity increased hyperbolically with increasing concentration of cA, and saturation with cA was observed at molar ratios of cA:cB higher than 5. Therefore, subsequent experiments were performed at a molar ratio of cA:cB of 5. Under these conditions, NTA-Mo activity was linearly dependent on protein concentration in the range of 1 to 32 µg ml⁻¹, with an average specific activity of 5 µmol of NTA min⁻¹ mg of protein⁻¹ compared with 0.8 µmol of NTA min⁻¹ mg of protein⁻¹ in cell extract. The activity was increased neither by additions of 20% polyethylene glycol or sorbitol, which would increase ionic interactions between the two components, nor by addition of 250 mM potassium glutamate, which favors hydrophobic interactions.

Optimum activity of reconstituted NTA-Mo was dependent on the presence of the following cofactors and substrates: 2 mM NTA, 2 mM MgCl₂, 10 µM FMN, and 3.4 mM

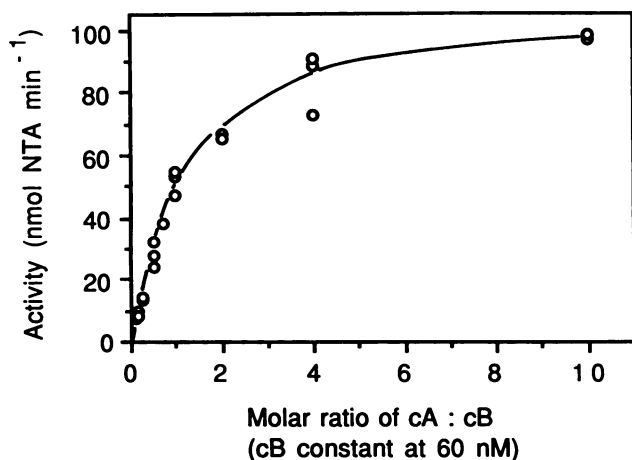


FIG. 4. Dependence of NTA-consuming activity of reconstituted NTA-Mo on the molar ratio of the two components, cA and cB. Each datum point represents the result of a single kinetic experiment. A constant concentration of cB (5 µg ml⁻¹) was used in all experiments, whereas the concentration of cA was varied.

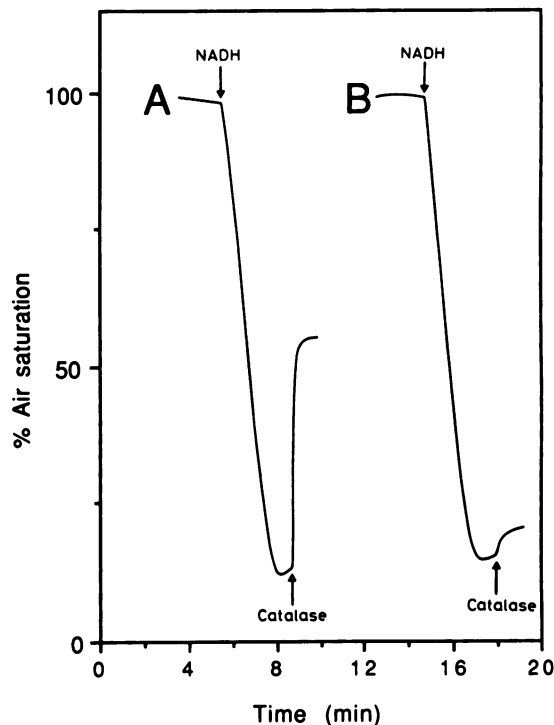


FIG. 5. Oxygen uptake experiments using NTA-Mo in a closed reaction vessel. Experiment A: Only cB (6 µg ml⁻¹) was added to the reaction mixture (2 mM NTA, 2 mM MgCl₂, 10 µM FMN, 50 mM TAPS [pH 8.5]). The reaction was started by the addition of 0.25 mM NADH at the time indicated. When all of the NADH was oxidized, 10 µg of catalase ml⁻¹ was added, and the formation of oxygen was monitored; experiment B: 80 µg of cA ml⁻¹ was present in addition to 6 µg of cB ml⁻¹. Otherwise, experimental conditions were identical to those for panel A.

NADH. MgCl₂ could be replaced by CoCl₂ or MnCl₂, but the activity was reduced to 80 or 70%, respectively, of that with MgCl₂. NADPH could not replace NADH, and omission of FMN or replacement of FMN by FAD resulted in a residual activity of 2% (when cB was not resaturated with FMN). The pH optimum of the purified and reconstituted NTA-Mo activity was shifted towards higher alkaline values compared with the result observed with cell extracts. Maximum activity of the reconstituted NTA-Mo was obtained at pH 8.5, and 35% activity remained at pH 7.0 and 9.6.

Dependence of uncoupling on cA concentration. One of the factors influencing the degree of coupling of NADH oxidation to NTA hydroxylation was the concentration of cA. The degree of coupling was experimentally assessed by monitoring both oxygen consumption and H₂O₂ formation in a closed reaction vessel fitted with an oxygen electrode. Two extreme conditions were compared: (i) coupling in the presence of cB only (6 µg ml⁻¹) and (ii) coupling in the presence of reconstituted NTA-Mo (7.5 µg of cB ml⁻¹ and 80 µg of cA ml⁻¹). As shown in Fig. 5, for the first case, all of the oxygen consumed was incorporated into H₂O₂. This was concluded from the fact that half of the oxygen consumed was released again after the addition of catalase. On the other hand, only 6% of the consumed oxygen was detected in H₂O₂ when reconstituted NTA-Mo was used. In order to test whether coupling of the reaction was dependent on the presence of cA, or whether uncoupling was prevented by the presence of other proteins, cA was replaced by either 200 µg of bovine

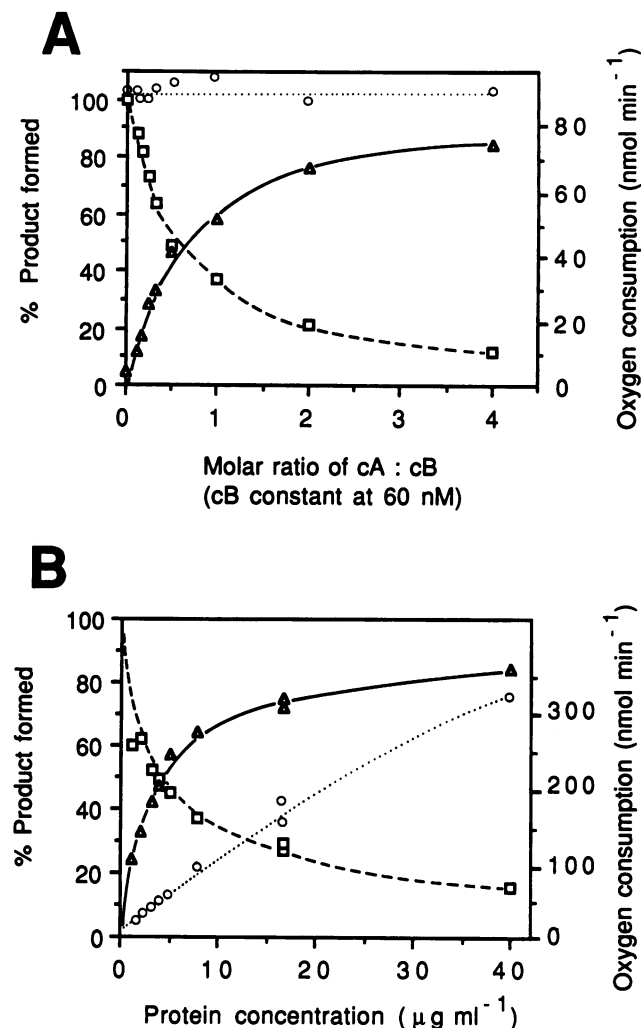


FIG. 6. Influence of the molar ratio of cA:cB (A) and the enzyme concentration (B) on the degree of coupling exhibited by reconstituted NTA-Mo. The relative proportions of H₂O₂ and glyoxylate formed are given as the percentages of the quantity of oxygen consumed. In all experiments, 5 μg of cB ml⁻¹ was used. ○, oxygen consumption rate; Δ, glyoxylate; □, H₂O₂. For panel B, the molar ratio of cA:cB was 1:1.

serum albumin ml⁻¹ or 200 μg of cytochrome *c* ml⁻¹. Neither BSA nor cytochrome *c* was able to circumvent uncoupling. Furthermore, neither cA on its own nor reconstituted NTA-Mo was able to oxidize H₂O₂.

In the same way, the influence of the molar ratio of cA:cB on uncoupling of the reaction was investigated in detail (Fig. 6A). At the end of the reaction, both H₂O₂ and glyoxylate were determined. The fraction of glyoxylate formed increased hyperbolically with increasing cA concentration, whereas the fraction of H₂O₂ formed decreased correspondingly. The rate of oxygen consumption was independent of the ratio of cA:cB. In all experiments, the sum of glyoxylate and H₂O₂ formed corresponded with the amount of oxygen consumed.

Dependence of uncoupling on NTA-Mo concentration. cA and cB were mixed at a molar ratio of 1:1, and the influence of protein concentration on the degree of uncoupling was investigated by using the same experimental setup as de-

TABLE 3. Inhibition of the NTA-monooxygenase

Control and added compound(s) ^a	Concn (mM)	Assay method ^b	Remaining activity (%)
Control ^c		NTA/O ₂	100
CaCl ₂	10.0	NTA	5
CaCl ₂ + NTA ^d	10.0	NTA	96
MgCl ₂ + acetamido-IDA ^d	10.0	NTA	96
EDTA	10.0	NTA	3
MgCl ₂ + EDTA ^d	10.0	NTA	84
MgCl ₂	10.0	NTA	91
FAD	0.1	NTA	24
FAD	0.1	O ₂	168
NaCl	250	NTA	54
NaCl	300	O ₂	96
DTT	10	O ₂	100
<i>p</i> -Hydroxymercuribenzoate	1	O ₂	12
<i>p</i> -Hydroxymercuribenzoate + 10 mM DTT	1	O ₂	96
5,5'-Dithio-bis-(2-nitrobenzoate)	1	O ₂	67
5,5'-Dithio-bis-(2-nitrobenzoate) + 10 mM DTT	1	O ₂	100

^a Compounds were added in addition to the compounds in the control experiment.

^b Activity was measured by either the NTA or O₂ consumption assay.

^c The control assay contained 2 mM NTA, 2 mM MgCl₂, 10 μM FMN, 50 mM TAPS (pH 8.5), and 3 mM NADH. The specific activities were 8.3 μmol of NTA min⁻¹ (mg of protein)⁻¹ in the NTA consumption assay and 6.6 μmol of O₂ min⁻¹ (mg of protein)⁻¹ in the oxygen consumption assay.

^d Both compounds were added in equimolar concentrations.

scribed in the previous section. The results (Fig. 6B) clearly demonstrate that at high protein concentrations, NADH oxidation is tightly coupled to the hydroxylation of NTA and the O₂ incorporated into glyoxylate increased hyperbolically with increasing concentrations of NTA-Mo, whereas at the same time, the fraction of H₂O₂ formed from O₂ decreased. However, the rate of oxygen consumed increased proportionally to the concentration of protein used.

Specificity of NTA-Mo. Of particular interest are the questions of whether a natural substrate exists for NTA-Mo and whether IDA is hydroxylated by NTA-Mo, as proposed by Firestone and coworkers (8). With respect to IDA, this compound was not used as a substrate by purified and reconstituted NTA-Mo, even at high concentrations of IDA (10 mM) and in the presence of equimolar concentrations of either MgCl₂ or MnCl₂. Furthermore, IDA did not stimulate NADH oxidation. The following compounds were tested for their ability to stimulate NADH oxidation (spectrophotometric assay) in the complete reaction mixture, *N*-methyl-IDA, *N*-acetamido-IDA, *N*-hydroxyethyl-IDA, EDTA, trimethylamine, triethanolamine, dimethylamine, dimethylglycine, methylamine, sarcosine, anthranilate, betaine, *N*-methylglutamate, *N*-methylalanine, *N*-methylaspartate, *N*-acetylglutamate, *N*-methylphenylalanine, *N*-methylleucine, *N*-methyltryptophane, glutamate, aspartate, citrate, proline, imidazoleacetate, asparagine, and ethylenediamine-*N,N'*-diacetate. In all instances, the rate of NADH oxidation was less than 2% of the rate observed with NTA.

Inhibition of NTA-Mo. Inhibition experiments were carried out at a cA concentration of 18 μg ml⁻¹ and a cB concentration of 6 μg ml⁻¹, and the results are summarized in Table 3. All the compounds tested were added to the complete reaction mixture used in the control experiment. In order to assess whether inhibition of NTA consumption activity was only due to increased uncoupling, some inhibi-

tion experiments were also carried out by using the oxygen consumption assay. Activity of NTA-Mo was inhibited by FeCl_2 and CaCl_2 but was not inhibited when the cations were complexed with NTA. Similarly, activity was inhibited by EDTA but not by Mg^{2+} -complexed EDTA. This suggests that the reduction in activity was caused by the reduced availability of Mg^{2+} -complexed NTA. Ca^{2+} -complexed NTA or Mg^{2+} -complexed acetamido-IDA, two compounds structurally similar to Mg^{2+} -complexed NTA, neither inhibited NTA consumption nor served as a substrate for NTA-Mo.

Furthermore, the enzyme was inhibited by the two thiol reagents hydroxymercuribenzoate and dithio-bis-(nitrobenzoate). The inhibition by the thiol reagents was reversible by addition of excess DTT. This would be consistent with the increased half-life time of cB in the presence of DTT.

NTA consumption was reduced to roughly 50% in the presence of 250 mM NaCl, KCl, or sodium acetate. In contrast, O_2 consumption was not inhibited up to 300 mM NaCl, indicating that high ionic strength resulted in uncoupling of NADH oxidation from NTA hydroxylation. This was experimentally confirmed in the oxygen measurement reaction vessel, where the proportion of H_2O_2 formed increased from 26% of consumed oxygen in the absence of NaCl to 70% in the presence of 250 mM NaCl. A similar effect was observed for FAD. Addition of 100 μM FAD at the same time reduced the rate of NTA consumption but stimulated O_2 consumption. Addition of FAD also led to a fourfold-increased proportion of H_2O_2 formed.

Kinetic parameters of NTA-Mo. K_m values were obtained from Lineweaver-Burk plots of initial reaction rates. In these experiments, the rate of NTA consumption was assessed via the rate of glyoxylate formation. The same K_m value of 0.5 mM \pm 0.15 mM was obtained both for NTA at a constant concentration of 2 mM MgCl_2 and for equimolar mixtures of MgCl_2 and NTA. The K_m value for FMN was found to be 1.3 μM \pm 0.2 μM , as determined by using cB containing approximately 0.1 mol of FMN per mol of native cB in the complete assay mixture. For the determination of the K_m value for NADH, the initial rates of NADH oxidation were measured. A value of 350 μM \pm 120 μM was obtained. The K_m value for oxygen (<25 μM) was too low to be determined with the experimental setup used.

DISCUSSION

The purification of the two components (cA and cB) of NTA-Mo was possible by using both an assay based on the consumption of NTA and the traditional spectrophotometric assay. Reconstitution of the functional complex from the purified components made it possible to characterize the enzyme. The complete NTA-Mo (both components and FMN) was found to be a classical monooxygenase that catalyzes the oxidation of NADH, with one oxygen atom appearing in the hydroxylated product (finally glyoxylate) and the other atom appearing in H_2O . However, incorporation of ^{18}O into glyoxylate cannot be demonstrated quantitatively because of the rapid exchange of the aldehyde oxygen with oxygen from H_2O . The fact that NTA-Mo did not accept any other structurally related compound as a substrate indicates that it has a restricted specificity and that it is not a broad-spectrum monooxygenase as, e.g., the pig liver flavin-containing monooxygenase (36). NTA-Mo is a two-component flavin monooxygenase and should be assigned the name nitrilotriacetate monooxygenase

(NTA, NADH:oxygen oxidoreductase; α -hydroxylating) and classified in EC group 1.14.13.

Firestone and coworkers (8) proposed that α -hydroxylated NTA is formed in the reaction, rather than NTA-N-oxide. Because NTA-N-oxide is a stable compound, its formation would imply the presence of a second enzyme for the transformation of NTA-N-oxide to glyoxylate and IDA. However, NTA-N-oxide was transformed neither in cell extracts nor by a partially purified, active NTA-Mo preparation (8).

All the data obtained support the scheme shown in Fig. 2 for both the catalytic activity of the functional NTA-Mo and the uncoupling of the NADH oxidation from hydroxylation of NTA by cB. Uncoupling is a well-known property of flavin monooxygenases, especially when substrate analogs are supplied (7, 19–21, 32, 35), but has also been reported for iron-sulfur-containing monooxygenases (11, 12, 34). cB of NTA-Mo, on its own, behaved in a manner similar to that of other flavin monooxygenases with substrate analogs, except that uncoupling was complete. When cA was also present, NTA was hydroxylated, but at present, the catalytic role of cA is unknown. Two roles for cA can be envisaged. (i) cA could be the hydroxylating component and cB functions as an NADH:cA oxidoreductase, or (ii) cA could modify the active site on cB in a way such that NTA is accepted as a substrate and becomes hydroxylated. The former mechanism has been described for 2,5-diketocamphane monooxygenase from *Pseudomonas putida* ATCC 17453 (27). However, in the case of NTA-Mo, such a mechanism seems unlikely because cA does not contain any flavin and it has been shown that a hydroperoxyflavin is involved in hydroxylation of the substrate in flavin monooxygenases (3). NTA-Mo, reconstituted with FMN-saturated cB and cA, was able to hydroxylate NTA without addition of FMN. However, the fact that a threefold stimulation of activity was observed after addition of 1 μM FMN (data not shown) indicates that in NTA-Mo flavin is only weakly bound or that a second weak FMN-binding site might exist. An alternative role of cA is that cA acts as a modifier of the monooxygenase reaction, as has been reported for cB of the three-component methane monooxygenase system from two type II methanotrophs (11, 12). cB of methane monooxygenase couples the reaction of the NADH reductase (containing FAD and an iron sulfur center) to the hydroxylase (containing an oxo- or hydroxo-bridged binuclear iron cluster). In the absence of cB, methane is inefficiently hydroxylated and a four-electron reduction of O_2 to H_2O has been postulated by Green and Dalton (12), whereas the formation of an unknown activated oxygen species has been suggested by Fox and coworkers (11).

Several in vitro properties of the enzyme complex suggest that the two components of NTA-Mo are only weakly associated. (i) They are easily separated by gel filtration, and (ii) activity increased hyperbolically with increasing cA concentrations with a half-saturation value of 2.5 molecules of cA per molecule of cB (Fig. 4). However, the observation that increasing concentrations of NTA-Mo (at equimolar concentrations of cA and cB) resulted in a more efficient hydroxylation of NTA (Fig. 6B) suggests that uncoupling is a result of in vitro conditions. This is supported by data from the immunquantification of the two components in cell extracts, in which it was found that they were present at approximately equimolar concentrations and in which NTA-Mo accounted for some 7% of the total cellular protein. Assuming a cell volume of approximately 1 μm^3 , the concentration of NTA-Mo can be estimated to be 5 mg ml^{-1} .

Thus, the in vivo concentration of NTA-Mo is expected to be 100 times higher than the protein concentrations tested experimentally. This suggests that the in vivo uncoupling is insignificant, even at the high salt concentrations of 150 to 300 mM potassium glutamate encountered in the cytoplasm (2).

Both high concentrations of salts and FAD favor uncoupling of the NTA-Mo reaction. Similar effects have been described for an FMN-containing 2,5-diketocamphane 1,2-monooxygenase (27). When the apoenzyme was saturated with FAD instead of FMN, NADH oxidation was uncoupled from hydroxylation of the natural substrate. Similarly, Entsch and coworkers (7) reported that phenol hydroxylase containing 1-deaza-FAD instead of FAD exhibited uncoupling, indicating that hydroperoxy-1-deaza-FAD was unfavorable for hydroxylation of phenol. Different salts have been reported to affect the interaction of FAD with phenol hydroxylase, leading to uncoupling (18). In analogy, the increased uncoupling of the NTA-Mo at high salt concentrations might be due to a disturbance of the interaction of FMN and the enzyme.

The results reported suggest that Mg^{2+} (Co^{2+} or Mn^{2+})-complexed NTA is the substrate for NTA-Mo (NTA stability constants, $\log K$, for Mg^{2+} , Mn^{2+} , and Co^{2+} are 5.4, 7.4, and 10.4, respectively). For example, sensitivity to EDTA was completely reversible by addition of excess $MgCl_2$, indicating that the addition of EDTA resulted in the removal of Mg^{2+} ions from NTA. Similarly, addition of 10 mM $CaCl_2$ (stability constant, $\log K$, for Ca^{2+} is 6.4) resulted in a reduction of the concentration of Mg^{2+} -complexed NTA, leading to a decrease in activity. This inhibition was reversed by addition of excess NTA. This proposal is also supported by the fact that the K_m values for Mg^{2+} and Mg^{2+} -complexed NTA are the same. Whether Mg^{2+} (Co^{2+} or Mn^{2+}) is involved in the oxidation reaction of NTA-Mo remains to be elucidated.

Firestone and coworkers reported that Mn^{2+} -complexed IDA was a substrate for their partially purified NTA-Mo (8). With the purified NTA-Mo described here, this activity was not detected. Therefore, another enzyme responsible for the subsequent metabolic transformation of IDA must exist in "*Chelatobacter*" strain ATCC 29600. Recently, we have been able to demonstrate the conversion of IDA to glyoxylate in a membrane preparation of "*Chelatobacter*" strain ATCC 29600 (4).

The question of whether there exists a yet-unknown natural substrate for NTA-Mo or whether NTA-Mo is a result of an evolutionary adaptation to this xenobiotic compound remains to be elucidated. Preliminary results obtained in our laboratory indicate that the presence of NTA-Mo is widespread among obligately aerobic NTA-degrading bacteria and that the enzyme is inducible during growth with NTA as a substrate (33). The initial results of McFeters and coworkers demonstrated that either induction or derepression of NTA-Mo must also occur during growth under complex environmental conditions (17). In their experiments, pure cultures of cells of *Chelatobacter* strain ATCC 29600, pregrown in the laboratory on complex medium in the absence of NTA (and therefore uninduced for NTA degradation), were transferred into sterile membrane diffusion chambers. Subsequent exposure of the chambers in a sewage treatment plant receiving NTA concentrations of approximately 1 mg liter⁻¹ led to the synthesis of NTA-degrading enzymes within several hours.

ACKNOWLEDGMENTS

We are indebted to G. Hamer, E. Kellenberger, and T. Bickle for encouragement and many stimulating discussions and to C. A. Mason for help during the preparation of the manuscript. We are grateful to F. Bonhote, St. Mattmüller, and E. Grollimund for help with chromatography and the possibility of analyzing IDA in the laboratory of Sarasin AG in Basel. Special thanks to L. V. Bystriykh of the University of Groningen for analysis of flavin and to H. Seiler and T. Eiche of the Institute of Anorganic Chemistry at Basel University for analysis of iron. We thank D. Egli for technical assistance and I. Thoenen for preparation of antisera. We are indebted to A. Lustig of the Department of Biophysics of the Biozentrum at Basel University for doing the analytical ultracentrifugation runs.

R.S. and T.U. were supported by a grant from Lever AG Switzerland and Unilever, Merseyside, United Kingdom. Additional financial support came from the Research Commission of ETH Zürich (to R.S.) and from the Kanton Basel-Stadt (to T.U.).

REFERENCES

1. Cripps, R. E., and A. S. Noble. 1973. The metabolism of nitrilotriacetate by a Pseudomonad. *Biochem. J.* **136**:1059-1068.
2. Csonka, L. N. 1989. Physiological and genetic responses of bacteria to osmotic stress. *Microbiol. Rev.* **53**:121-147.
3. Cummings Reyerson, C., D. P. Ballou, and C. Walsh. 1982. Mechanistic studies on cyclohexanone oxygenase. *Biochemistry* **21**:2644-2655.
4. Egli, T., M. Bally, and T. Uetz. 1990. Microbial degradation of chelating agents used in detergents with special reference to nitrilotriacetic acid (NTA). *Biodegradation* **1**:121-132.
5. Egli, T., H.-U. Weilenmann, T. El-Banna, and G. Auling. 1988. Gram-negative, aerobic, nitrilotriacetate-utilizing bacteria from wastewater and soil. *Syst. Appl. Microbiol.* **10**:297-305.
6. El-Banna, T., G. Auling, E. Wilberg, and T. Egli. Submitted for publication.
7. Entsch, B., M. Husain, D. P. Ballou, V. Massey, and C. Walsh. 1980. Oxygen reactivity of hydroxybenzoate hydroxylase containing 1-deaza-FAD. *J. Biol. Chem.* **255**:1420-1429.
8. Firestone, M. K., S. D. Aust, and J. M. Tiedje. 1978. A nitrilotriacetic acid monooxygenase with conditional NADH-oxidase activity. *Arch. Biochem. Biophys.* **190**:617-623.
9. Firestone, M. K., and J. M. Tiedje. 1978. Pathway of degradation of nitrilotriacetate by a *Pseudomonas* species. *Appl. Environ. Microbiol.* **35**:955-961.
10. Focht, D. D., and H. A. Joseph. 1971. Bacterial degradation of nitrilotriacetic acid. *Can. J. Microbiol.* **17**:1553-1556.
11. Fox, B. G., W. A. Froland, J. E. Dege, and J. D. Lipscomb. 1989. Methane monooxygenase from *Methylosinus trichosporium* OB3b. *J. Biol. Chem.* **264**:10023-10033.
12. Green, J., and H. Dalton. 1985. Protein B of soluble methane monooxygenase from *Methylococcus capsulatus* (Bath). *J. Biol. Chem.* **260**:15795-15801.
13. Harlow, E., and D. Lane. 1988. *Antibodies: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. Kaki, K., H. Yamaguchi, Y. Iguchi, M. Teshima, T. Shirakashi, and M. Kuriyama. 1986. Isolation and characteristics of nitrilotriacetate-degrading bacteria. *J. Ferment. Technol.* **64**:103-108.
15. Keesey, J. 1987. *Biochimica information*. Boehringer Mannheim Biochemicals, Indianapolis, Ind.
16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
17. McFeters, G. A., T. Egli, E. Wilberg, A. Alder, R. P. Schneider, M. Snozzi, and W. Giger. 1990. Activity and adaptation of nitrilotriacetate (NTA)-degrading bacteria: field and laboratory studies. *Water Res.* **24**:875-881.
18. Neujahr, H. Y. 1983. Effect of anions, chaotropes, and phenol on the attachment of flavin adenine dinucleotide to phenol hydroxylase. *Biochemistry* **22**:580-584.
19. Neujahr, H. Y., and A. Gaal. 1975. Phenol hydroxylase from yeast: sulfhydryl groups in phenol hydroxylase from *Trichosporon cutaneum*. *Eur. J. Biochem.* **58**:351-357.
20. Powlowski, J. B., S. Dagley, V. Massey, and D. P. Ballou. 1987.

- Properties of anthranilate hydroxylase (deaminating), a flavo-protein from *Trichosporon cutaneum*. *J. Biol. Chem.* **262**:69–74.
21. Ribbons, D. W., and Y. Ohta. 1970. Uncoupling of electron transport from oxygenation in the mono-oxygenase, orcinol hydroxylase. *FEBS Lett.* **12**:105–108.
 22. Schachman, H. K. 1959. *Ultracentrifugation in biochemistry*. Academic Press, New York.
 23. Schneider, R. 1989. The NTA-monoxygenase from *Pseudomonas* ATCC 29600. Ph.D. thesis. Swiss Federal Institute of Technology, Zürich, Switzerland.
 24. Schneider, R., F. Zürcher, T. Egli, and G. Hamer. 1989. Ion chromatography method for iminodiacetic acid determination in biological matrices in the presence of nitrilotriacetic acid. *J. Chromatogr.* **462**:293–301.
 25. Schneider, R. P., F. Zürcher, T. Egli, and G. Hamer. 1988. Determination of nitrilotriacetate in biological matrices using ion exclusion chromatography. *Anal. Biochem.* **173**:278–284.
 26. Snozzi, M., and T. Egli. 1987. Purification of an NTA-monoxygenase, p. 345. *In* O. M. Neijssel et al. (ed.), *Proceedings of the Fourth European Congress on Biotechnology*, vol. 3. Elsevier, Amsterdam.
 27. Taylor, D. G., and P. W. Trudgill. 1986. Camphor revisited: studies of 2,5-diketocamphane 1,2-monoxygenase from *Pseudomonas putida* ATCC 17453. *J. Bacteriol.* **165**:489–497.
 28. Tiedje, J. M. 1980. Nitrilotriacetate: hindsight and gunsight, p. 114–119. *In* A. M. Maki, K. L. Dickson, and J. Cairns (ed.), *Biotransformation and fate of chemicals in the aquatic environment*. American Society for Microbiology, Washington, D.C.
 29. Tiedje, J. M., B. B. Mason, C. B. Warren, and E. J. Malek. 1973. Metabolism of nitrilotriacetate by cells of *Pseudomonas* species. *Appl. Microbiol.* **25**:811–818.
 30. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354.
 31. Trijbels, F., and G. D. Vogels. 1966. Degradation of allantoin by *Pseudomonas acidovorans*. *Biochim. Biophys. Acta* **113**:292–301.
 32. Tsuji, H., T. Ogawa, N. Bando, and K. Sasaoka. 1986. Purification and properties of 4-aminobenzoate hydroxylase, a new monoxygenase from *Agaricus bisporus*. *J. Biol. Chem.* **28**:13203–13209.
 33. Uetz, T., and T. Egli. Unpublished data.
 34. Wende, P., F. H. Bernhardt, and K. Pfeleger. 1989. Substrate-modulated reactions of putidamonoxin. *Eur. J. Biochem.* **181**:189–197.
 35. White-Stevens, R. H., and H. Kamin. 1972. Studies of a flavo-protein, salicylate hydroxylase. I. Preparation, properties, and the uncoupling of oxygen reduction from hydroxylation. *J. Biol. Chem.* **247**:2358–2370.
 36. Ziegler, D. M. 1990. Flavin-containing monoxygenases: enzymes adapted for multisubstrate specificity. *Trends Pharmacol. Sci.* **11**:321–324.