# Expression and purification of the recombinant subunits of toluene/ *o*-xylene monooxygenase and reconstitution of the active complex

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This paper describes the cloning of the genes coding for each component of the complex of toluene/*o*-xylene monooxygenase from *Pseudomonas stutzeri* OX1, their expression, purification and characterization. Moreover, the reconstitution of the active complex from the recombinant subunits has been obtained, and the functional role of each component in the electron transfer from the electron donor to molecular oxygen has been determined.

The coexpression of subunits B, E and A leads to the formation of a subcomplex, named H, with a quaternary structure (BEA)$_2$, endowed with hydroxylase activity.

Tomo F component is an NADH oxidoreductase. The purified enzyme contains about 1 mol of FAD, 2 mol of iron, and 2 mol of acid labile sulfide per mol of protein, as expected for the presence of one [2Fe–2S] cluster, and exhibits a typical flavodoxin absorption spectrum.

Interestingly, the sequence of the protein does not correspond to that previously predicted on the basis of DNA sequence. We have shown that this depends on minor errors in the gene sequence that we have corrected.

C component is a Rieske-type ferredoxin, whose iron and acid labile sulfide content is in agreement with the presence of one [2Fe–2S] cluster. The cluster is very sensitive to oxygen damage.

Mixtures of the subcomplex H and of the subunits F, C and D are able to oxidize *p*-cresol into 4-methylcatechol, thus demonstrating the full functionality of the recombinant subunits as purified.

Finally, experimental evidence is reported which strongly support a model for the electron transfer. Subunit F is the first member of an electron transport chain which transfers electrons from NADH to C, which tunnels them to H subcomplex, and eventually to molecular oxygen.

*Keywords*: monooxygenase; protein expression; electron transfer; bioremediation; recombinant.

Several strains from *Pseudomonas* genus grow on aromatic compounds due to enzymatic systems able to activate aromatic rings by mono- and di-hydroxylations and to operate *ortho* or *meta*-cleavage pathway [1,2] which leads to citric acid cycle intermediates.

Toluene/*o*-xylene-monooxygenase (Tomo) from *Pseudomonas stutzeri* OX1 [3,4] is endowed with a broad spectrum of substrate specificity [3], and the ability to hydroxylate more than a single position of the aromatic ring in two consecutive monooxygenation reactions [3]. Thus Tomo is able to oxidize *o*-, *m*- and *p*-xylene, 2,3- and 3,4-dimethylphenol, toluene, cresols, benzene, naphthalene, ethylbenzene, styrene [3], trichloroethylene, 1,1-dichloroethylene, chloroform [5] and tetrachloroethylene [6]. This makes the complex unique with respect to other known monooxygenases, such as toluene/benzene-2-monooxygenase from the *Pseudomonas* sp. strain JS150 [7], toluene-3-monooxygenase from *Pseudomonas pickettii* PKO1 [8], toluene-4-monooxygenase (T4MO) from *Pseudomonas mendocina* KR1 [9], and toluene-2-monooxygenase (T2MO) from *Burkholderia cepacia* G4 [10], and potentially useful for its use in bioremediation strategies [5,6,11] and/or the synthesis of commercially valuable compounds [12].

The genes coding for toluene/*o*-xylene monooxygenase have been cloned in pGEM 3Z vector (pBZ1260) [3]. The nucleotide sequence revealed six ORFs, named *tou A, B, C, D, E* and *F* (*tou*, for toluene/o-xylene utilization), which showed relevant similarities to the subunits of several enzymatic complexes involved in the oxygenation of aromatic compounds [4]. On the basis of homology studies of the coding gene sequence [4] it has been hypothesized that the gene products of the cluster form an electron transfer complex in which Tomo F, an NADH-oxidoreductase, is the first member of the electron transport chain. Tomo F is able to transfer electrons from NADH to Tomo C, which is a Rieske-type ferredoxin that tunnels electrons to the terminal oxygenase, the Tomo H subcomplex composed by the *tuoA*,

*touB* and *touE* gene products. Finally, another member of the complex is subunit Tomo D, for which a regulatory function has been suggested [4,13].

The present study reports the cloning, expression and purification of the individual components of Tomo in *Escherichia coli*, and their reconstitution into a functional complex. Subunits Tomo A, B, C, D and E were expressed in soluble form, while subunit Tomo F was expressed as an insoluble product, renatured *in vitro*, and purified. To our knowledge, this is the first example of a flavodoxin refolded from inclusion bodies.

## MATERIALS AND METHODS

### Materials

Bacterial cultures, plasmid purifications and transformations were performed according to Sambrook [14]. Double stranded DNA was sequenced with the dideoxy method of Sanger [15], carried out with the Sequenase version II Sequencing Kit and labeled nucleotides from Amersham. pET22b(+) expression vector and *E. coli* strain BL21DE3 were from Novagen, whereas *E. coli* strain JM101 was purchased from Boehringer. The thermostable recombinant DNA polymerase used for PCR amplification was PLATINUM Pfx from Life Technologies, and deoxynucleotide triphosphates were purchased from Perkin-Elmer Cetus. The Wizard PCR Preps DNA Purification System for elution of DNA fragments from agarose gel was obtained from Promega. Enzymes and other reagents for DNA manipulation were from New England Biolabs. The oligonucleotides were synthesized at the Stazione Zoologica 'A. Dohrn' (Naples, Italy). Poly(vinylidene difluoride) (PVDF) membranes were from Perkin Elmer Cetus. Protease inhibitor cocktail EDTA-free tablets were purchased from Boehringer. Superose 12 PC 3.2/30, Q-Sepharose Fast Flow, Sephacryl S300 High Resolution and Sephadex G75 Superfine, and disposable PD10 desalting columns were from Pharmacia. DEAE-Cellulose DE52 was from Whatman, CNBr was from Pierce, cytochrome *c* from horse heart, trypsin and bovine insulin from Sigma. All other chemicals were from Sigma. Tomo D subunit was expressed and purified as described [13]. The expression and purification of catechol 2,3-dioxygenase from *P. stutzeri* OX1 will be described in a different paper (Viggiani, manuscript in preparation).

### Construction of expression vectors

The individual genes *tou A, B, C, D, E* and *F* were obtained by PCR amplification of the DNA coding for the complex (GenBank, accession number AJ005663) cloned into plasmid pGEM 3Z (pBZ1260) [3], kindly supplied by P. Barbieri (Dipartimento di Biologia Strutturale e Funzionale, Università dell'Insubria, Varese, Italy). Synthetic oligonucleotide primers were designed to insert the appropriate endonuclease restriction sites at the 5′ and 3′ ends of each gene to allow their polar cloning into pET22b(+) expression vector.

The DNA fragments coding for Tomo C and Tomo B from the PCR amplifications were isolated by agarose gel electrophoresis, eluted and digested with *Nde*I and *Hin*dIII restriction endonucleases. The digestion products were purified by electrophoresis, ligated with pET22b(+) previously cut with the same enzymes, and used to transform JM101 competent cells. The resulting recombinant plasmids, named pET22b(+)/touC and pET22b(+)/touB, were verified by DNA sequencing.

pET22b(+)/touBEA plasmid coding for the three subunits B, E and A was obtained by inserting *touA* and *touE* genes into plasmid pET22b(+)/touB. This vector was first subjected to oligonucleotide mediated site-directed mutagenesis according to Kunkel [16] to remove an *Xho*I internal restriction site and to allow cloning of *touE* and *touA* genes at the 3′ end of the *touB* gene. For this purpose, the *touE* sequence was subjected to PCR mutagenesis to insert a *Not*I site at its 5′ end and an *Eco*RI site followed by an *Xho*I site at its 3′ end. The mutagenized DNA fragment was isolated by agarose gel electrophoresis, eluted and digested with *Not*I and *Xho*I restriction endonucleases. The digestion product was purified by electrophoresis, ligated with mutagenized pET22b(+)/touB previously cut with *Not*I and *Xho*I, and used to transform JM101 competent cells. The resulting plasmid was then cut with *Eco*RI and *Xho*I and ligated with *touA*, previously mutagenized by a PCR procedure to insert an *Eco*RI site at its 5′ end and a *Xho*I site at its 3′ end, and digested with the same enzymes. The final product was named pET22b(+)/touBEA.

When the DNA coding for Tomo F cloned into plasmid pGEM 3Z (pBZ1006) [4] was sequenced (GenBank accession number AJ438269), we did not find an A at position 6987, in accordance with the previously published sequence (GenBank accession number AJ005663). This difference generates a frame shift in our sequence which eliminates the stop codon formerly present at nucleotide 7042 (nucleotide numbering is given with reference to the sequence present in the GenBank at accession number AJ005663), and locates a new stop codon at nucleotide 7070. Moreover, at nucleotide 6851 was found to be a G instead of a C. The DNA coding for Tomo F cloned into plasmid pGEM 3Z (pBZ1006) was subjected to site-directed mutagenesis by PCR using two specific synthetic oligonucleotides to insert at the 5′ and 3′ ends the appropriate endonuclease restriction sites (*Eco*RI and *Nde*I at the 5′, and *Hin*dIII at the 3′) to allow cloning into pUC118 and pET22b(+).

The resulting fragment was purified by agarose gel electrophoresis, digested with *Eco*RI and *Hin*dIII, cloned into pUC118 previously cut with the same enzymes, and used to transform JM101 competent cells. This recombinant plasmid was then subjected to a second round of site-directed mutagenesis according to Kunkel [16], to remove an internal *Nde*I restriction site. This was done to allow *touF* cloning into the *Nde*I site of the expression vector pET22b(+). The coding sequence was then removed from pUC118 using *Nde*I and *Hin*dIII and subcloned in pET22b(+) digested with the same enzymes, and purified. The sequence of the resulting plasmid, named pET22b(+)/touF, was verified by DNA sequencing.

### Expression of recombinant plasmids

Plasmids pET22b(+)/touBEA, /touC and touF, were expressed using *E. coli* BL21DE3 cells.

All recombinant strains were routinely grown in LB medium [14] supplemented with 50 $\mu g \cdot mL^{-1}$ ampicillin. Fresh BL21DE3 transformed cells were inoculated into 10 mL of LB/ampicillin medium, at 37 °C, up to

$D_{600} = 0.7$. These cultures were used to inoculate 1 L of LB supplemented with 50 $\mu g \cdot mL^{-1}$ ampicillin, and grown at 37 °C until $D_{600}$ ranged from 0.7 to 0.8.

Expression of recombinant proteins was induced by adding isopropyl thio-β-D-galactoside at a final concentration of 25 $\mu M$ for pET22b(+)/touBEA, 0.4 mM for pET22b(+)/touC and 0.1 mM for pET22b(+)/touF. For plasmids pET22b(+)/touBEA and /touC, at the time of induction $Fe(NH_4)_2(SO_4)_2$ in $H_2SO_4$ was added at a final concentration of 100 $\mu M$. Growth continued for 3 h at 37 °C in the case of pET22b(+)/touC, and at 25 °C in the case of pET22b(+)/touBEA and /touF. The cells were harvested, washed with buffer A (25 mM Mops, pH 6.9, containing 10% (v/v) ethanol, 5% (v/v) glycerol, 0.08 M NaCl and 2 mM dithiothreitol), collected by centrifugation and the cell paste stored at −80 °C until needed.

An SDS/PAGE analysis of an aliquot of induced and noninduced cells, after sonication and separation of the soluble and insoluble fractions, revealed (data not shown) that based on the expected molecular size of the polypeptides, all the proteins of interest were present in the soluble fraction of the induced cell in the case of the expression of pET22b(+)/touBEA and /touC, whereas the product of the expression of pET22b(+)/touF was accumulated in the insoluble fraction, presumably as inclusion bodies.

The proteins were identified by N-terminal sequencing on samples blotted directly on PVDF membranes from electrophoresis gels. This confirmed that all the proteins were the mature products of the corresponding genes.

Typical yields, on the basis of a densitometric scanning of the electrophoresis profiles obtained after cell lysis, were approximately 20–30 $mg \cdot L^{-1}$ for Tomo C, 300 $mg \cdot L^{-1}$ for Tomo F, and 100 $mg \cdot L^{-1}$ for the expression products of pET22b(+)/touBEA.

## Preparation of the soluble fraction from transformed cells

The paste from 1 L culture of BL21DE3 cells transformed with pET22b(+)/touC and pET22b(+)/touBEA was suspended in 40 mL of buffer A containing an EDTA-free protease inhibitor cocktail. Cells were disrupted by sonication (10 × 1 min cycle, on ice). Cell debris was removed by centrifugation at 18 000 *g* for 60 min at 4 °C. The supernatant was immediately fractionated as described below.

## Purification of Tomo C

Unless otherwise stated all chromatographic steps were performed at 4 °C. Buffers were made anaerobic by repeated cycles of flushing with nitrogen. Column operations were not strictly anoxic.

The soluble fraction from a 2-L culture of cells expressing plasmid pET22b(+)/touC was loaded onto a Q-Sepharose Fast Flow column (1 × 18 cm) equilibrated in buffer A at a flow rate of 10 $mL \cdot h^{-1}$, and the column was further washed with 50 mL of the same buffer. Proteins were eluted using a 300-mL linear salt gradient from 0.15 to 0.4 M NaCl in buffer A, at a flow rate of 10 $mL \cdot h^{-1}$. Fractions eluting at about 0.35 M NaCl were found to contain Tomo C, as evidenced by UV/VIS absorption at 280 and 460 nm, SDS/PAGE analysis, and N-terminal sequencing of the electro-

phoresis band electroblotted onto PVDF membranes [17] (data not shown). Fractions eluting at 0.35 M NaCl were pooled, concentrated by ultrafiltration on YM3 membranes, and loaded onto a Sephadex G75 Superfine column (2.5 × 50 cm) equilibrated in buffer A containing 0.3 M NaCl, at a flow rate of 12 $mL \cdot h^{-1}$. The ferredoxin peak was concentrated by ultrafiltration on YM3 membranes, diluted threefold with buffer A, loaded again onto the Q-Sepharose Fast Flow column, and eluted using the same procedure described above. Fractions containing electrophoretically pure Tomo C were pooled, purged with $N_2$ and stored at −80 °C. A molar extinction coefficient at 458 nm was determined among several preparations, and found to be 6870 ± 130 $M^{-1} \cdot cm^{-1}$. This value is in good agreement with those reported for other Rieske-type ferredoxins [18,19]. Final yield was about 4 mg of protein from a 2-L culture. Figure 1 shows an SDS/PAGE analysis of purified Tomo C.

Tomo C preparations can be stored under a nitrogen barrier at −80 °C at least for 8 months without any damage, whereas storage at +4 or −20 °C leads to the loss of their spectral properties in few days.

## Purification of the expression products of pET22b(+)/touBEA

The soluble fraction from a 1-L culture of cells expressing plasmid pET22b(+)/touBEA was loaded onto a Q-Sepharose Fast Flow column (1 × 18 cm) equilibrated in buffer A at a flow rate of 10 $mL \cdot h^{-1}$. The column was washed further with 50 mL of the same buffer. Elution was performed using a 300-mL linear salt gradient from 0.08–0.35 M NaCl in buffer A, at a flow rate of 10 $mL \cdot h^{-1}$. An SDS gel electrophoresis of the fractions
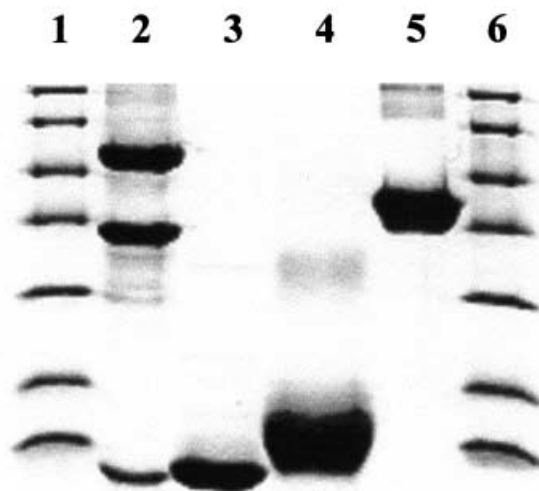


**Fig. 1. SDS/PAGE analysis of Tomo purified subunits.** Lanes 1 and 6, molecular mass standards (β-galactosidase, 116.0 kDa, BSA, 66.2 kDa, ovalbumin, 45.0 kDa, lactate dehydrogenase, 35.0 kDa, restriction endonuclease Bsp981, 25.0 kDa, β-lactoglobulin, 18.4 kDa, lysozyme, 14.4 kDa). Lane 2, Tomo H (7 μg); lane 3 Tomo D (5 μg); lane 4 Tomo C (5 μg); Lane 5, Tomo F (6 μg).

eluted from the column indicated that fractions eluting at 0.3 M NaCl contained three polypeptides with an apparent molecular mass of about 10, 38 and 57 kDa, the expected molecular size of recombinant subunits B, E and A, respectively. The identity of the proteins was further checked by N-terminal sequencing of the electrophoresis bands electroblotted onto PVDF membranes [17], by their comparison with the sequences expected from the translation of the coding genes. Relevant fractions were pooled and concentrated by ultrafiltration on YM30 membrane, then loaded onto a Sephacryl S300 High Resolution column (2.5 × 50 cm) equilibrated in buffer A containing 0.3 M NaCl, at a flow rate of 6 mL·h$^{-1}$. Also on this chromatographic matrix the three proteins coeluted in a single peak containing Tomo B, E and A polypeptides. Fractions were pooled, concentrated by ultrafiltration on YM30, and stored under nitrogen at −80 °C. The final yield was about 20 mg of proteins per litre of culture. The SDS/PAGE analysis of the complex is shown in Fig. 1.

### In vitro renaturation and purification of recombinant Tomo F

To isolate inclusion bodies, cells from 1 L of culture were suspended in 20 mL of 50 mM Tris/acetate, pH 8.4, and sonicated (10 × 1 min cycle, on ice). The suspension was then centrifuged at 18 000 *g* for 30 min at 4 °C. In order to remove membrane proteins, the cell pellet was washed twice in 0.1 M Tris/acetate, pH 8.4, containing 4% (v/v) Triton X-100 and 2 M urea, followed by repeated washes in water, to eliminate traces of Triton and urea. Clean inclusion bodies were then stored at − 20 °C as dry pellet until use.

For *in vitro* renaturation of Tomo F, 10 mg of inclusion bodies were dissolved at a final concentration of 2 mg·mL$^{-1}$ in 0.1 M Tris/HCl, pH 8.4, containing 6 M guanidine/HCl and 20 mM dithiothreitol, purged with $O_2$-free nitrogen and incubated for 3 h at 37 °C. The sample was then diluted 20-fold in 100 mL (final volume) of a refolding buffer containing 0.1 M Tris/HCl pH 7.0, 0.5 M L-arginine, 50 μM FAD, 10 μM ferrous ammonium sulfate, 10 μM sodium sulfide, 2 mM dithiothreitol and 0.3 M guanidine/HCl, at a final protein concentration of 0.1 mg·mL$^{-1}$. After 1 h at room temperature, the mixture was extensively dialyzed at 4 °C against 50 mM Tris/HCl pH 7.0, containing 5% (v/v) glycerol and 1 mM dithiothreitol. The sample was then concentrated by ultrafiltration on a YM30 membrane. Any insoluble material was removed by centrifugation, and the supernatant was then loaded onto a DEAE-Cellulose DE52 column (0.5 × 10 cm) equilibrated in buffer A (25 mM Mops, pH 6.9, containing 10% (v/v) ethanol, and 5% (v/v) glycerol). The column was washed at a flow rate of 10 mL·h$^{-1}$ with 20 mL of buffer A, and elution was carried out stepwise with 20 mL of buffer A containing 0.1, 0.3 and 0.8 M NaCl, respectively. The fractions eluted at 0.1 M NaCl contained Tomo F, as shown by SDS/PAGE analysis (data not shown). They were pooled and loaded onto a PD-10 gel filtration column (1.6 × 5 cm) equilibrated in 50 mM Tris/HCl, pH 7.0, containing 5% (v/v) glycerol and 0.25 M NaCl, at a flow rate of 2 mL·min$^{-1}$. This last purification step was necessary to

remove unincorporated FAD or any other small molecules such as iron and sulfur before protein characterization. The protein peak was purged with $N_2$ and stored at −80 °C. Typical yields were 3–4 mg of Tomo F starting from 10 mg of inclusion bodies. The SDS/PAGE analysis of purified Tomo F is shown in Fig. 1.

A molar extinction coefficient at 454 nm was determined among several preparations, and found to be 48 100 ± 500 M$^{-1}$·cm$^{-1}$.

### Expression and preparation of recombinant apo-Tomo F

Expression and preparation of recombinant apo-Tomo F, devoid of the [2Fe−2S] center, was obtained using the same procedures described for recombinant Tomo F except for the presence of 5 mM EDTA in all the steps of the renaturation and purification procedures to chelate iron and prevent cluster formation.

### Enzymatic assays of Tomo F reductase activity

NADH acceptor reductase activity of Tomo F was assayed spectrophotometrically using Tomo C as electron acceptor. Assays were performed at 25 °C by adding Tomo F (0.02–8 μg) to 0.4 mL of a solution containing 25 mM Mops, pH 6.9, 5% (v/v) glycerol, 10% (v/v) ethanol, 0.1 M NaCl, 60 μM NADH (or NADPH) and 20 μM Tomo C. Activity was measured by recording the decrease in absorbance at 458 nm, using a Δε value of 3095 ± 105 M$^{-1}$·cm$^{-1}$, the difference between the extinction coefficient of oxidized and reduced Tomo C, one unit of activity being the μmoles of reduced Tomo C formed per min at 25 °C.

### Multiple turnover assays for the reconstituted Tomo complex

All assays were performed at 25 °C in 0.1 M Tris/HCl, pH 7.5. Tomo activity was assayed by determining the 4-methylcatechol (4-MC) produced by oxidation of *p*-cresol. 4-MC amount was measured in a coupled assay with recombinant catechol 2,3-dioxygenase from *P. stutzeri* OX1 [20] (Viggiani, manuscript in preparation), which cleaves the 4-MC ring and produces 2-hydroxymuconic semialdehyde. This can be monitored at 410 nm (ε = 12 620 M$^{-1}$·cm$^{-1}$).

The assay mixture contained, in a final volume of 400 μL, 0.1 M Tris/HCl, pH 7.5, 1 mM NADH, 1 mM *p*-cresol, saturating amounts of catechol 2,3-dioxygenase and the four Tomo components. Component concentrations were 0.15 μM Tomo H, 0–1.2 μM Tomo F, 0–3 μM Tomo C and 0–3 μM Tomo D.

Assay mixtures were prepared with all components, except for subunit Tomo F, and the reaction was initiated by the addition of this latter recombinant subunit. The absorbance increase at 410 nm was then followed for 5 min. Specific activity was expressed as nanomoles of *p*-cresol converted per min per mg of complex at 25 °C.

It should be added that controls were run to check the presence of saturating amounts of NADH over the reaction time. This was done by running duplicate assays and monitoring the absorbance at 340 nm (the reduced NADH absorption maximum), and at 410 nm. NADH concentration was estimated using an extinction coefficient of 6.22 mM$^{-1}$·cm$^{-1}$.

Kinetic parameters were determined by the program GRAPHPAD PRISM (http://www.graphpad.com).

### Single turnover assay

Single turnover assays of the individual components (10 nmol of Tomo H, 20 nmol of Tomo C and Tomo D subunits) and of each of their possible combinations, were performed by adding the proteins to reaction mixtures containing 0.1 M Tris/HCl, pH 7.5, and 1 mM p-cresol, in a final volume of 200 μL. Anaerobiosis was established by repeated cycles of flushing and filling with nitrogen. Fully reduced proteins were obtained by the addition of sodium dithionite in a 10-fold molar excess relative to the concentration of Tomo A, in the presence of 50 μM methyl viologen as a redox mediator. Reactions were started by air injection and vigorous mixing, and then incubated for 3 min at 25 °C. To measure the amount of 4-MC obtained from p-cresol oxidation, each sample was first diluted twofold with 200 μL of water, and used to record the baseline. Saturating amounts of catechol 2,3-dioxygenase were then added, and the spectrum recorded after 5 min of incubation. The total amount of hydroxymuconic semialdehyde was calculated by its absorption at 382 nm ($\varepsilon = 28\,100$ $M^{-1}{\cdot}cm^{-1}$), after baseline subtraction.

### Protein sequencing and mass spectrometry

Protein sequencing, electrospray mass spectrometric measurements, and MALDI mass spectrometry (MALDI/MS) analysis of peptide mixtures was performed as already described [13].

### Iron and labile sulfide determination

Total iron content was determined colorimetrically by complexation with Ferene S [10], or Ferrozine [21].

Inorganic sulfide content was determined by methylene blue formation as described by Rabinowitz [22] and Brumby [23], with a minor modification of the incubation time with the alkaline zinc reagent, which was extended to 2 h.

### Extraction and identification of FAD from TomoF

Flavin content of Tomo F was calculated spectrophotometrically after heat denaturation of the protein. Enzyme solutions were kept in boiling water for 3 min, the resulting precipitate was removed by centrifugation, and the spectrum of the supernatant recorded. Flavin cofactor concentration was estimated using an extinction coefficient of 11.3 $mM^{-1}{\cdot}cm^{-1}$, at 450 nm.

Flavin identity was confirmed by reverse phase HPLC of the supernatant on a $C_{18}$–silica column. The sample was loaded on the column equilibrated in 2% acetonitrile in water containing 0.1% (v/v) trifluoroacetic acid, and washed for 10 min in the same solvent. Elution was carried out using an isocratic elution with 8% (v/v) acetonitrile in water containing 0.1% (v/v) trifluoroacetic acid. The identification of the flavin cofactor was obtained by comparing the retention time of the eluted peak with that of reference samples of authentic FAD and FMN.

### Tomo C reduction by sodium dithionite

Reduction of Tomo C was obtained by the anaerobic addition of a 100-fold excess of sodium dithionite with respect to the protein. Sodium dithionite was prepared as a 100-mM solution in 25 mM Mops, pH 6.9.

### Separation of the subunits of the subcomplex H

Subunits B, E and A from Tomo H were separated by HPLC using a Phenomenex Jupiter narrow bore $C_4$ column (2.1 × 250 mm, 300 Å pore size), at a flow rate of 0.2 mL·min$^{-1}$ with a linear gradient of a two-solvent system. Solvent A was 0.1% (v/v) trifluoroacetic acid in water, solvent B was acetonitrile containing 0.07% (v/v) trifluoroacetic acid. Proteins were separated by a multistep gradient of solvent B from 10–40% in 40 min followed by 10 min isocratic elution, from 40–50% in 40 min.

### Estimation of molecular mass by gel filtration

Determination of the molecular mass was performed by gel filtration on a Superose 12 PC 3.2/30 (3.2 mm × 300 mm) column equilibrated in 25 mM Mops, pH 6.9, containing 0.2 M NaCl, using a SMART-System (Pharmacia Biotech). The molecular mass markers used as standards for gel filtration chromatography were β-amylase (200 kDa), aspartate aminotransferase (90 kDa), ribosome inactivating protein (29 kDa) and onconase (11.8 kDa).

### Other methods

SDS/PAGE was carried out according to Laemmli [24]. Protein concentrations were determined colorimetrically with the Bradford Reagent [25] from Sigma, using 1–10 μg BSA as a standard. N-terminal protein sequence determinations were performed on an Applied Biosystems sequenator (model 473A), connected online with an HPLC apparatus for identification of phenylthiohydantoin derivatives. Amino-terminal sequencing was carried out on polypeptides separated by denaturing gel electrophoresis and then electroblotted onto PVDF membranes [17].

## RESULTS AND DISCUSSION

### Characterization of recombinant Tomo C

When recombinant Tomo C was analyzed by electrospray mass spectrometry, the protein was found to possess a molecular mass of 12 372.7 ± 0.9 Da, consistent with that of mature Tomo C with six free sulfydryls, whose theoretical molecular mass is 12 372.8 Da, as calculated on the basis of the amino acid sequence deduced by the nucleotide sequence.

The primary structure of recombinant Tomo C was verified by peptide mapping. Aliquots of the HPLC purified protein were digested with trypsin and the resulting peptide mixtures were analyzed by MALDI/MS. The mass signals recorded in the spectra were mapped onto the anticipated sequence of subunit C on the basis of their mass value and the specificity of the enzyme, leading to the complete verification of the amino acid sequence of subunit C (GenBank accession number AJ005663).

Tomo C solutions, colored in brown-orange, showed an absorbance spectrum with four maxima at 278, 323, 458 and 560 nm (Fig. 2A) consistent with the presence of a Rieske-type [2Fe−2S] center. Among several preparations of purified Tomo C, the ratio of $A_{458} : A_{278}$ was always found to be higher than 0.21, in agreement with the data reported for T4MOC [26] and for the Rieske iron–sulfur protein from *Thermus thermophilus* [19]. The inset of Fig. 2A shows also the spectrum of the reduced form of Tomo C, obtained by reduction with sodium dithionite. The absorbance at 458 nm decreased by about 50%, whereas two new maxima appeared at 420 nm and 520 nm. Tomo C was found to be reversibly reoxidized in the presence of air (Fig. 2A, inset). The spectrum of the oxidized form of Tomo C did not change in presence of stoichiometric amounts of Tomo D
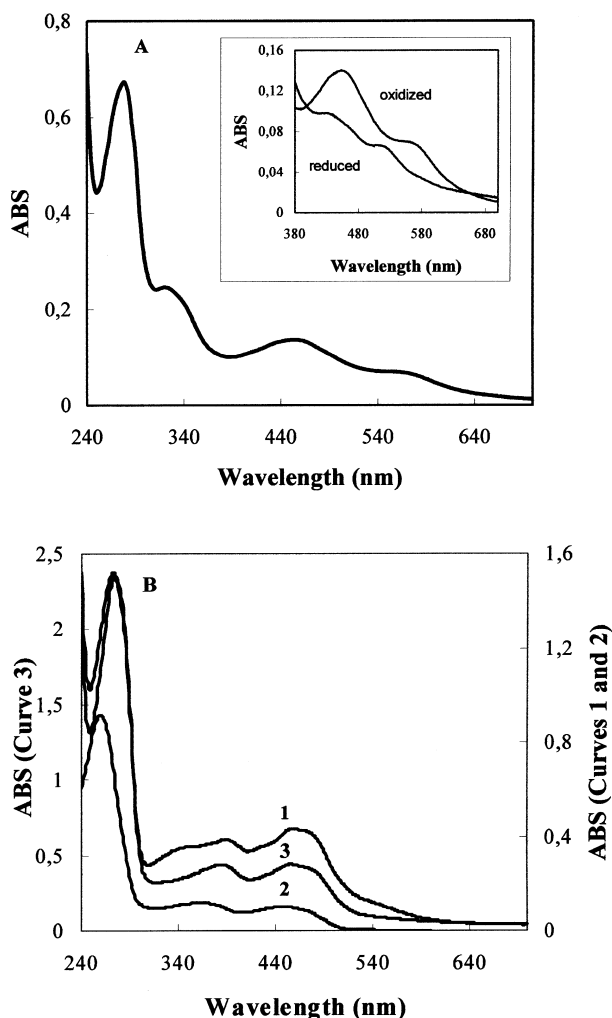
and Tomo H or substoichiometric amounts of Tomo F. The effect on Tomo C of equimolar amounts of Tomo F could not be investigated because this subunit absorbs in the same spectral region of Tomo C.

Iron content was determined to be 1.6–1.8 mol·mol$^{-1}$ of protein, while acid-labile sulfide content was found to be 1.8–2.1 mol·mol$^{-1}$ of protein. Thus, we can confidently conclude that recombinant Tomo C contains one Rieske-type [2Fe−2S] center per enzyme molecule.

## Characterization of recombinant Tomo F

Samples of purified subunit F were subjected to electrospray mass spectrometry. The average molecular mass value measured for Tomo F was 38 044.03 ± 1.6 Da. This value is in good agreement with the theoretical value calculated on the basis of the deduced amino acid sequence of subunit F lacking the initial methionine residue (38 043.5 Da).

The primary structure of the recombinant subunit F of Tomo was verified by the same strategy used for Tomo C.

The protein is 9 residues longer than the sequence predicted on the basis of the translation of the *touF* gene (GenBank accession number AJ005663), thus confirming the corrections we have inserted in that sequence and reported in GenBank at accession number AJ438269.

The UV/VIS spectrum of purified Tomo F (curve 1 of Fig. 2B) shows absorbance maxima around 273, 335, 385 and 454 nm, with shoulders at 425 and 480 nm as already reported for other oxidoreductases from several complexes [10,18,27–29]. Moreover, $A_{273} : A_{454}$ ratios determined over several Tomo F preparations ranged from 3.5 to 3.9, in agreement with data collected for phthalate oxygenase reductase from *Pseudomonas cepacia* and for phenol hydroxylase from *Acinetobacter radioresistens* [21,30].

When the enzyme solution was heated to 100 °C, the spectrum recorded for the soluble fraction was that of free FAD, as shown in Fig. 2B (curve 2). This was confirmed by HPLC analysis carried out as described in Materials and methods. Quantitative analysis of bound FAD yielded the value of 1.1–1.2 mol of FAD per mole of protein.

The iron content of Tomo F was 1.8–2.1 mol·mol$^{-1}$ of protein, and the acid-labile sulfide content was found to be between 2 and 2.3 mol·mol$^{-1}$ of protein.

Therefore we can confidently conclude that Tomo F contains one [2Fe−2S] center and one FAD molecule.

The specific activity of Tomo F measured using Tomo C subunit as a specific acceptor was found to be 73.6 ± 2.3 U·mg$^{-1}$. It should be noted that the activity of the protein is strictly dependent on the presence of the iron center. In fact, when apo-Tomo F (which contains FAD) was used as a catalyst in the same assay, no activity was detected. This indicates that the lack of the [2Fe−2S] cluster prevents electron transfer from NADH to the acceptor, which confirms the role of the iron sulfur cluster as the redox mediator between FAD and the iron center. The lack of the cluster in apo-Tomo F was confirmed also by the spectrum of the protein (Fig. 2B, curve 3), which is that typical of a flavoprotein with maxima at 273, 390 and 450 nm, and a shoulder at 480 nm [29].

Furthermore, the specific activity of a different type of recombinant Tomo F, expressed in a soluble form using pBZ1260 expression vector [3] was also measured, and found to be about 50 U·mg$^{-1}$. This value is almost identical



**Fig. 2. Absorption spectra of (A) purified oxidized and reduced Tomo C and (B) recombinant Tomo F.** (A) Absorption spectrum of purified oxidized Tomo C (23 μM) in buffer A containing 0.3 M NaCl. The inset shows the spectra of sodium dithionite reduced (23 μM), and air reoxidized Tomo C. (B) Absorption spectrum of: curve 1, recombinant (0.34 mg·mL$^{-1}$) Tomo F; curve 2, flavin nucleotide dissociated from recombinant Tomo F after heat denaturation as described in the text; curve 3, apo-Tomo F (0.45 mg·mL$^{-1}$). Samples were all dissolved in 50 mM Tris/HCl, pH 7.0, containing 5% (v/v) glycerol and 0.25 M NaCl.

to that measured for recombinant Tomo F renatured *in vitro* following the procedure described in the present paper. This result strongly supports the idea that *in vitro* renatured Tomo F is functionally identical to naturally folded Tomo F.

The ability of Tomo F to use either NADH or NADPH as electron donors was also measured. The specific activity with NADPH was $0.718 \pm 0.09$ U·mg$^{-1}$, i.e. about 100-fold lower than that determined using NADH as electron donor. These values, while confirming that Tomo F can use either NADH or NADPH, indicate that the protein is specific for NADH, in line with the results obtained with other oxygenases [27,31,32].

The ability of recombinant Tomo F to transfer electrons from NADH to Tomo C was also studied, measuring the effect (a) on the Tomo F spectrum after the addition of NADH, and (b) on the Tomo C spectrum after the addition of NADH followed by the addition of Tomo F.

When recombinant Tomo F was incubated (Fig. 3, curve 1), with an eightfold excess of NADH, progressive changes in its spectral properties were observed. The spectra were recorded up to 15 min. After 1 min (Fig. 3, curve 2) a decrease in absorbance at 454 nm (about 52% of the initial value) was recorded, and three new maxima appeared at 534, 583 and 640 nm, with an isosbestic point at 518 nm. As shown in Fig. 3 curve 3, the spectrum closely resembles those reported for other reductases in their reduced form [27,28], in which the increase in absorbance between 520 nm and 700 nm has been ascribed to FAD reduction [27,28]. At about 3 min NADH was found to be almost completely reoxidized, as indicated by the disappearance of the peak at 340 nm. From this time on, a progressive increase of the absorbance at 454 nm and a concomitant absorption decrease in the range 520–700 nm was recorded, which
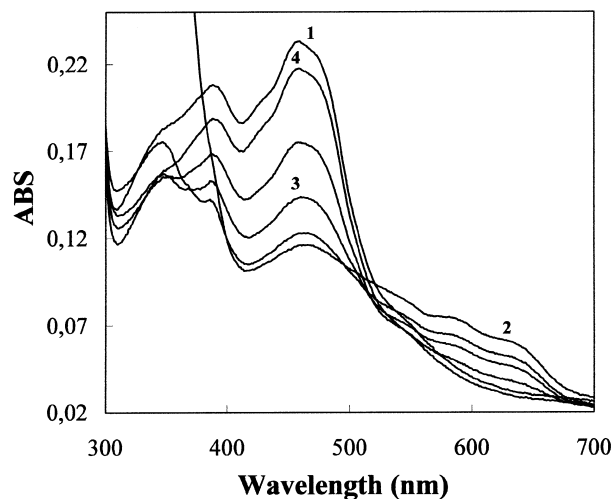


**Fig. 3. Reduction of recombinant Tomo F by NADH.** Spectra were recorded at the times indicated below upon the addition of NADH (final concentration 37.4 μM) to a solution of recombinant Tomo F (4.7 μM) dissolved in 50 mM Tris/HCl, pH 7.0, containing 5% (v/v) glycerol and 0.25 M NaCl. Curve 1, spectrum of oxidized Tomo F before addition of NADH; curve 2, 1 min; curve 3, 3 min; curve 4, 15 min. Curves not labeled with numbers have been recorded between 3 and 15 min after NADH addition.

can be ascribed to the reoxidation of Tomo F by oxygen in solution. After 15 min (Fig. 3, curve 4) the spectrum became almost that of oxidized Tomo F. This indicates that the reversible transfer of electrons was complete.

As for the transfer of electrons from recombinant Tomo F to Tomo C, curve 1 in Fig. 4 shows the spectrum of Tomo C in which the typical spectrum of the oxidized form is evident [19,33], with absorbance maxima at 278, 323, 458 and 560 nm. NADH addition did not change the spectrum (Fig. 4, curve 2), which indicates the inability of Tomo C to accept electrons directly from NADH. Addition of recombinant Tomo F to the mixture induces a decrease in the absorbance between 400 and 600 nm, with a shift of the peaks at 458 and 560 nm to 420 and 520 nm, respectively, characteristic of the reduced form of Tomo C [19,33].

The maximum decrease in absorbance was monitored after 1 min (Fig. 4, curve 3). After 7 min (Fig. 4, curve 4) the disappearance of the peak at 340 nm was observed, due to the complete NADH oxidation, with the gradual shift of the peaks at 420 and 520 nm to 458 and 560 nm, respectively, thus indicating the reoxidation of Tomo C. After 11 min (Fig. 4, curve 5) the typical spectrum of oxidized Tomo C was recorded, due to the transfer of electrons to oxygen.

These data give a direct evidence of the direction of the electron transfer from Tomo F to Tomo C.

## Characterization of Tomo H subcomplex

*Expression, purification and quaternary structure studies.* A comparison of the deduced amino acid sequences of the six ORFs of the *tou* gene cluster from *P. stutzeri* OX1 with the counterparts found in databases led us to assign a putative function to each component of the multicomponent monooxygenase system [3,4]. These studies led to the hypothesis that subunits B, E and A might constitute a subcomplex, endowed with hydroxylase activity, as occurs in other monooxygenase complexes [7,9,10,18,34].

The purification procedure of the proteins expressed by plasmid pET22b(+)/touBEA showed that Tomo B, E and A coeluted in a single peak in all the chromatographic systems. As these included ion-exchange and gel filtration chromatography, and the proteins were expected to have different isoelectric points and different molecular masses (10, 38 and 57 kDa, respectively), these results suggest the association of the polypeptides in a complex.

The protein mixture derived from the last gel filtration step of the purification procedure was then subjected to molecular mass determination by gel filtration on a Superose 12 PC 3.2/30. The apparent molecular mass was found to be 206 kDa. This value is consistent with the hypothesis that the three proteins associate to form a stable complex, named Tomo H, whose quaternary structure is (BEA)$_2$, similar to other hydroxylase complexes of monooxygenases [18,33,34].

Samples of purified Tomo H subcomplex were analyzed by LC/MS. Components B, E and A showed molecular mass of $9841.6 \pm 0.6$ Da, $38\ 201.4 \pm 2.9$ Da and $57\ 591.5 \pm 3.6$ Da, respectively. These values are in good agreement with the expected molecular mass calculated on the basis of the deduced amino acid sequence of the mature
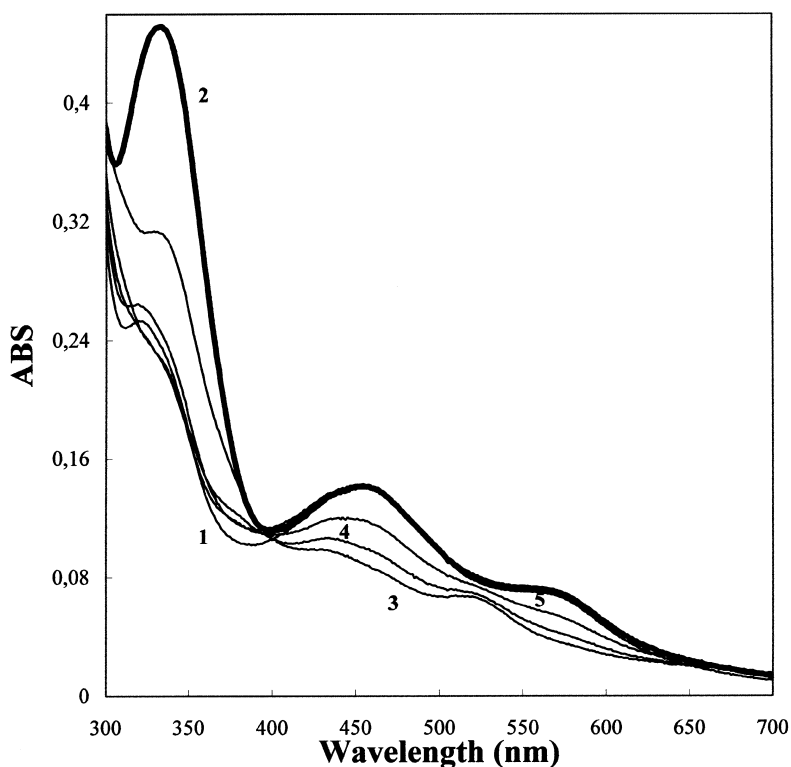
**Fig. 4. Reduction of Tomo C by recombinant Tomo F and NADH.** Curve 1, spectrum of a solution (23.5 μM) of Tomo C in 25 mM Mops, pH 6.9, containing 1% (v/v) glycerol, 2% (v/v) ethanol and 0.06 M NaCl. Curve 2 (bold line), same as curve 1, upon addition of NADH (final concentration 23.5 μM). Curve 3, same as curve 2 immediately after addition of 0.32 μg of recombinant Tomo F (16.7 nM). Spectra recorded after 7 min (curve 4) and 11 min (curve 5) after recombinant Tomo F addition are also shown.

form of the subunits (B, 9842.2 Da; E, 38 202.9 Da and A, 57 593.7 Da).

The primary structure of the recombinant subunits of Tomo H subcomplex was verified by peptide mapping as described for subunit C. The results led to the complete verification of the amino acid sequence of subunits B, E and A, demonstrating that the subunits of the recombinant complex Tomo H have the amino acid sequence predicted on the basis of the corresponding DNA sequences, as present in the GenBank at the accession number AJ005663.

Finally, the iron content of the complex was determined and found to be 3.4 mol·mol$^{-1}$ of Tomo H. This result is in agreement with the presence of a diiron center in each of the subunit Tomo A, as suggested by its homology with other monooxygenases 'large' subunit [33–35].

Moreover, the absorption spectrum of purified recombinant Tomo H is featureless above 300 nm. The lack of absorption in the visible region suggests that Tomo H has a hydroxo-bridged diiron center similar to that described for methane monooxygenase hydroxylase complex from *Methylococcus capsulatus* [34], alkene monooxygenase from *Nocardia corallina* B-276 [12] and for T4MO [33], rather than an oxo-bridged diiron center [36].

## Reconstitution of the Tomo complex from recombinant subunits

Functional characterization of the recombinant subunits of the complex of toluene/*o*-xylene monooxygenase was carried by testing their ability to reconstitute a functional complex, i.e. the ability to catalyze the conversion of a substrate into a product, mediated by electrons coming from the donor NADH.

Preliminary multiple-turnover activity assays indicated that mixtures of equimolar amounts of the purified Tomo components were able to transform *p*-cresol into 4-MC.

To determine the optimal relative concentration of each subunit in order to obtain maximum hydroxylase activity we carried out kinetic measurements using mixtures of Tomo H, F, C and D, and changing the concentration of each single component.

Figure 5 shows the effects on the rate of reaction of increasing ratios of Tomo F, Tomo C and Tomo D with respect to Tomo H in the presence of constant amounts of the other components. A linear relationship is obtained at low ratios of Tomo C and D followed by a sharp break at about 1.6 mol of Tomo C per mol of Tomo H and 3 mol of Tomo D per mol of Tomo H (Fig. 5A,B), respectively. The nearly linear titration and the break is an indication of a high affinity of these components for Tomo H as already observed for the regulatory component of methane monooxygenase (MMO) [37], and suggests the existence of a stable complex between Tomo H, Tomo C and Tomo D with a possible stoichiometry of 1 : 2 : 2 (relative to Tomo H).

Tomo F instead shows a different behavior. In fact, the maximum velocity is reached at substoichiometric amounts of this component with respect to Tomo H (about 0.2 mol of Tomo F per mol of Tomo H), and no titration break is present (Fig. 5C). These results would suggest that Tomo F, unlike Tomo C and D, does not form a stable complex with Tomo H, as observed for the reductase component of MMO [37].

Based on the information above, we measured the kinetic parameters of the reconstituted complex using saturating ratios of the components. The value of the specific activity was 380 ± 30 nmol of *p*-cresol converted per min per mg of
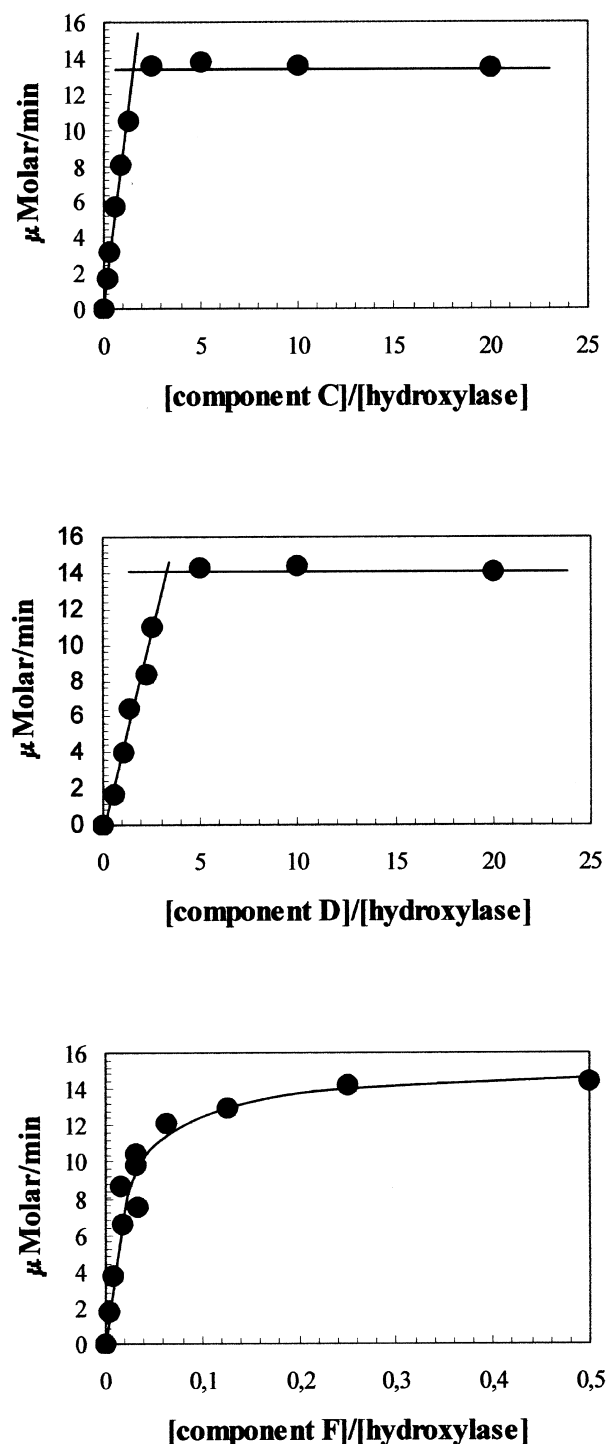
**Fig. 5. The effect of different ratios of Tomo C (A), Tomo D (B) and Tomo F (C) components with respect to the hydroxylase on the rate of toluene/*o*-xylene monooxygenase.** Activity was measured as described in Materials and methods. Curve A: Tomo H, 0.15 μM; Tomo D, 0.75 μM; Tomo F, 0.075 μM. Curve B: Tomo H, 0.15 μM; Tomo C, 0.75 μM; Tomo F, 0.075 μM. Curve C: Tomo H, 0.15 μM; Tomo C and D, 0.75 μM.

Tomo H, whereas $k_{cat}$ and $K_m$ values were $0.62 \pm 0.02$ s$^{-1}$ and $13.3 \pm 1.3$ μM, respectively. It should be noted that the $K_m$ value is in good agreement with that determined using

*E. coli* cells expressing the entire Tomo complex from vector pBZ1260 ($19.4 \pm 2$ μM).

Thus, we can confidently conclude that the recombinant components expressed and purified with the procedures described above are able to reconstitute an active Tomo complex in which all the individual subunits are functional.

To identify the hydroxylase component of the complex we performed single-turnover assays, in the absence of the Tomo F subunit, by measuring the ability of Tomo H, Tomo C and Tomo D, in each possible combination, to oxidize *p*-cresol to 4-MC.

The results of the experiments carried out as described in Materials and methods, using sodium dithionite as a reductant and methyl viologen as a redox mediator, are reported in Table 1. They clearly indicate that only Tomo H by itself is able to convert *p*-cresol, thus strongly supporting its identification with the hydroxylase component of the complex, in agreement with the hypothesis based on homology studies [4].

Data of Table 1 also indicate that addition of Tomo C or Tomo D to Tomo H increases the amount of the product of 2.3- and 3.6-fold, respectively, with respect to that measured in their absence. Moreover, when all the three components were present, a 23-fold increase in the amount of the product, with respect to that produced in the presence of Tomo H alone, was recorded. This latter data is clear evidence of a cooperative interaction between the three components, suggestive of the formation of a ternary complex, as it has been demonstrated for other homologous monooxygenases [37,38].

As for the increase in the amount of 4-MC produced in the presence of both Tomo H and Tomo C, it may well be attributed to the ability of reduced Tomo C to transfer additional electrons to Tomo H, thus promoting more than one reaction cycle in the single turnover assay. This data, together with the observation that Tomo C can be reversibly reduced in the presence of Tomo F and NADH, strongly support the idea that Tomo C acts as a mediator in the electron transfer chain between Tomo F and Tomo H, in line with the hypothesis raised on the basis of homology studies [4].

As for Tomo D, a protein devoid of any redox center [13], the data of Table 1 support (although not conclusively) its regulatory role in the complex. In fact, the 3.6-fold increase in the ability of Tomo H to transform *p*-cresol into 4-MC, in the absence of any capability of Tomo D to transfer electrons, can be attributed to its capacity to modulate the activity of the hydroxylase component of the complex, as it

**Table 1. Single-turnover assays catalyzed by the components of the toluene/*o*-xylene monooxygenase complex.** The experiments were performed as described in Materials and methods using 10 nmol of Tomo H and 20 nmol of Tomo C and Tomo D.

| Components | *p*-cresol converted (nmol) |
|---|---|
| Tomo H | 0.075 |
| Tomo C | 0 |
| Tomo D | 0 |
| Tomo H + Tomo C | 0.173 |
| Tomo H + Tomo D | 0.271 |
| Tomo H + Tomo C + Tomo D | 1.72 |

has already been demonstrated for homologous proteins such as T4MOD of the T4MO from *P. mendocina* KR1 [33] and subunit B of methane monooxygenases [38,39].

Moreover, it should be noted that the omission of Tomo D in multiple-turnover assays leads to a complete absence of activity (data not shown) despite the presence of all the other components of the electron transport chain. This result is in line with the absence of any oxidase activity recorded in experiments carried out *in vivo* on *E. coli* cells harboring a cluster *tou* in which *touD* gene was inactivated by partial deletion [4]. However, it should be noted that this data does not parallel the effect of the absence of other homologous regulatory subunits of oxygenase complexes, like T4MOD [33] and component B of methane monooxygenases [38,39]. In these cases the absence of the regulatory subunit induces only a reduction of the hydroxylase activity.

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