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Stereospecific Hydroxylation of Indan by *Escherichia coli* Containing the Cloned Toluene Dioxygenase Genes from *Pseudomonas putida* F1

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***Escherichia coli* JM109(pDTG601), containing the *todC1C2BA* genes encoding toluene dioxygenase from *Pseudomonas putida* F1, oxidizes indan to (−)-(1*R*)-indanol (83% *R*) and *trans*-1,3-indandiol. Under similar conditions, *P. putida* F39/D oxidizes indan to (−)-(1*R*)-indanol (96% *R*), 1-indanone, and *trans*-1,3-indandiol. The differences in the enantiomeric composition of the 1-indanols formed by the two organisms are due to the presence of a 1-indanol dehydrogenase in *P. putida* F39/D that preferentially oxidizes (+)-(1*S*)-indanol.**

Pseudomonas putida F1 initiates the degradation of toluene by incorporating both atoms of oxygen into the aromatic nucleus to form (+)-*cis*-(1*R*,2*S*)-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol [5, 6]). The enzyme catalyzing this reaction, toluene dioxygenase, also catalyzes the benzylic oxidation of indan and indene to (−)-(1*R*)-indanol and (+)-(1*S*)-indenol, respectively (9). We have now reinvestigated the stereospecificity of indan oxidation by the toluene dioxygenase present in *P. putida* F39/D, a mutant strain of *P. putida* F1 that lacks the enzyme *cis*-toluene dihydrodiol dehydrogenase (5), and here we compare the results with those for *Escherichia coli* JM109(pDTG601), a recombinant organism that contains the structural genes (*todC1C2BA*) for toluene dioxygenase cloned in the vector pKK223-3 (10). The results show that differences in the enantiomeric composition of the (−)-(1*R*)-indanol formed by the two organisms are due to the presence of a dehydrogenase in *P. putida* F39/D which preferentially oxidizes (+)-(1*S*)-indanol to 1-indanone.

Cells of *E. coli* JM109(pDTG601) were grown with shaking (200 rpm) at 37°C on mineral salts basal medium (8) supplemented with 20 mM glucose, 1 mM thiamine, and ampicillin (100 μg/ml). When the culture reached a turbidity of 0.5 at 600 nm, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to give a final concentration of 1 mM. After 1 h, cells were harvested and suspended to a turbidity of 2.0 at 600 nm in 50 mM phosphate buffer, pH 7.2, containing 20 mM glucose. Cell suspensions, 50 ml in 250-ml Erlenmeyer flasks, were incubated at 30°C on a rotary shaker (200 rpm) in the presence of indan. After 6 h, the contents of each flask were extracted three times with equal volumes of ethyl acetate. The organic extracts were dried over anhydrous sodium sulfate and concentrated under vacuum at 30°C. The products formed were separated on a 25-m Hewlett Packard Ultra-1 nonpolar fused silica capillary column programmed from 80 to 150°C at 2°C/min and from 150 to 280°C at 20°C/min. The carrier gas was helium (25 cm s^{-1}), and detection was by a Hewlett Packard 5970 mass selective detector. All products were identified by their retention times and by showing that their mass spectra were identical to the spectra of authentic compounds. The major product formed from indan was 1-indanol (99%). A minor product (~1%) was identified as *trans*-1,3-indandiol. 1-Indanone was not detected. In control experiments, IPTG-treated cells of *E. coli* JM109(pKK223-3) did not oxidize indan. When the experiments described above were conducted with toluene-induced cells of *P. putida* F39/D, the products formed from indan were 1-indanol (88%), 1-indanone (11%), and *trans*-1,3-indandiol (~1%).

The enantiomeric composition of the 1-indanol formed from indan by *E. coli* JM109(pDTG601) and *P. putida* F39/D was determined by gas chromatography of its isopropyl urethane derivative (4, 7). Samples were dissolved in CH_2Cl_2, and the enantiomeric isopropyl urethanes were separated on a 50-m XE-60-(*S*)-valine-(*S*)-phenylethylamide fused silica column (7) at 170°C with helium as the carrier gas (25 cm s^{-1}). Under these conditions, the retention times for the (−)-(1*R*)-indanol and (+)-(1*S*)-indanol derivatives were 21.91 and 22.31 min, respectively. The results obtained showed that *E. coli* JM109(pDTG601) oxidized indan to (−)-(1*R*)-indanol (83% *R*) in contrast to the (−)-(1*R*)-indanol (96% *R*) formed by *P. putida* F39/D. The formation of almost enantiomerically pure 1-indanol by *P. putida* F39/D indicated that the 1-indanol dehydrogenase previously detected in this organism (9) preferentially oxidizes (+)-(1*S*)-indanol. This observation was confirmed by incubating toluene-induced cells of *P. putida* F39/D for 6 h with authentic (−)-(1*R*)-indanol (>97% *R*). Analysis of the reaction mixture revealed the presence of 1-indanol (85%), 1-indanone (11%), and *trans*-1,3-indandiol (4%). Gas chromatography of the isopropyl urethane derivative of the remaining (−)-(1*R*)-indanol showed that it consisted of more than 97% of the *R* enantiomer. In contrast, the same cell suspension oxidized (+)-(1*S*)-indanol completely to 1-indanone in 6 h. Analogous experiments with *E. coli* JM109(pDTG601) showed that *trans*-1,3-indandiol (10%) and a trace of 1-indanone (<1%) were the only products formed from (−)-(1*R*)-indanol, and approximately 90% of the added 1-indanol was recovered

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TABLE 1. Indanol dehydrogenase activity in cell extracts of *P. putida* F39/D[a]

Substrate	pH	Sp act[b]
(+)-(1*S*)-Indanol	7.2	0.052
(−)-(1*R*)-Indanol	7.2	<0.001
(+)-(1*S*)-Indanol	8.0	0.069
(−)-(1*R*)-Indanol	8.0	0.002

[a] Enzyme activities were determined by monitoring the increase in A_{340} due to the reduction of NAD. Substrates were present in saturating amounts, and activity was linear with respect to protein concentration. Reactions were initiated by the addition of either (+)-(1*S*)- or (−)-(1*R*)-indanol to the reaction mixture.

[b] Micromoles of NADH formed per minute per milligram of protein.

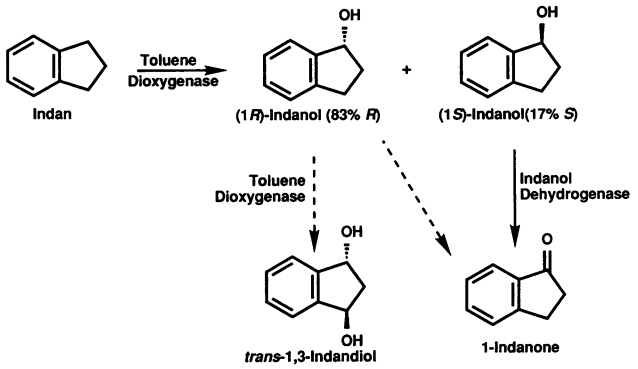

FIG. 1. Proposed reactions in the oxidation of indan to 1-indanol by toluene dioxygenase present in *E. coli* JM109(pDTG601) and *P. putida* F39/D. Also shown is the oxidation of (+)-(1*S*)-indanol to 1-indanone by a dehydrogenase present only in *P. putida* F39/D. The dehydrogenase shows slight activity with (−)-(1*R*)-indanol as indicated by the dashed line. The oxidation of (−)-(1*R*)-indanol to *trans*-1,3-indandiol is a minor reaction catalyzed by toluene dioxygenase in both organisms. The absolute stereochemistry of the indandiol was not determined.

from the reaction mixture. The recombinant *E. coli* did not oxidize (+)-(1*S*)-indanol (>98% *S*).

Cell extracts, prepared from toluene-induced cells of *P. putida* F39/D, were examined for their ability to oxidize (−)-(1*R*)- and (+)-(1*S*)-indanol in the presence of NAD^+ and $NADP^+$. The results obtained with NAD^+ as the electron acceptor are shown in Table 1. $NADP^+$ was less effective, giving rates three times lower than those obtained with NAD^+. The rate of oxidation of (−)-(1*R*)-indanol was negligible at pH 7.0, and at pH 8.0 it was 35 times lower than the rate observed with (+)-(1*S*)-indanol.

The results of the present study show that the differences in the enantiomeric composition of the 1-indanol formed from indan by *E. coli* JM109(pDTG601) and *P. putida* F39/D are due to the preferential oxidation of (+)-(1*S*)-indanol by a dehydrogenase present in the latter organism. The partial resolution of racemic 1-indanol into the *R* enantiomer by a different strain of *P. putida* (strain UV4) has been reported and is also attributed to the preferential oxidation of (+)-(1*S*)-indanol (2). In a previous report, we showed that *P. putida* F39/D oxidizes indan to (−)-(1*R*)-indanol (88 to 92% *R*). The reaction times in these experiments were limited to 1 h in order to minimize the effect of an inducible indanol dehydrogenase on the chirality of the 1-indanol formed from indan (9). However, in light of the present studies, it now appears that the enantiomeric composition of the 1-indanol observed previously was due to either partial resolution by the dehydrogenase and incomplete recovery of 1-indanone from large-scale reaction mixtures or the loss of (+)-(1*S*)-indanol during isolation, purification, and derivatization procedures. The in situ formation of the enantiomeric isopropyl urethane derivatives of 1-indanol and their resolution by gas chromatography on a chiral capillary column are significantly more sensitive and accurate than chiroptical methods and procedures involving the resolution of diastereomers (3). Consequently, the enantiomeric composition of the 1-indanol (83% *R*) formed by *E. coli* JM109 (pDTG601) reflects the true enantiospecificity of toluene dioxygenase.

The reaction products formed from indan by toluene dioxygenase are shown in Fig. 1. The formation of *trans*-1,3-indandiol has not been observed previously, and its absolute stereochemistry was not determined in the present study. However, it seems probable that, starting with (−)-(1*R*)-indanol, it would be the (1*R*,3*R*) enantiomer.

P. putida F39/D oxidizes indene to (+)-(1*S*)-indenol (63% *S*) and (−)-*cis*-(1*S*,2*R*)-indandiol (65% *S*,*R*) (9). These products, which have similar enantiomeric compositions, are also formed from indene by the cloned toluene dioxygenase in *E. coli* (data not shown). Thus, it is of interest to note that although *P. putida* UV4 oxidizes indene to (−)-*cis*-(1*S*,2*R*)-indandiol (60% *S*,*R*), the 1-indenol formed by this organism is the *R* enantiomer (99% *R*) (1). These observations suggest that there may be subtle but significant differences in the active sites of the toluene dioxygenases from *P. putida* F1 and *P. putida* UV4.

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