Evidence that Operons tcb, tfd, and clc Encode Maleylacetate Reductase, the Fourth Enzyme of the Modified ortho Pathway

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The maleylacetate reductase from Pseudomonas sp. strain B13 functioning in the modified ortho pathway was purified and digested with trypsin. The polypeptides separated by high-performance liquid chromatography were sequenced. Alignments with the polypeptides predicted from the tfdF and tcbF genes located on plasmids pJP4 of the 2,4-dichlorophenoxyacetate-degrading Alcaligenes eutrophus JMP134 and pP51 of the 1,2,4-trichlorobenzene-degrading Pseudomonas sp. strain P51 as well as polypeptides predicted from the tfE gene located on the chromosome of the 2,4,5-trichlorophenoxyacetate-degrading Burkholderia cepacia AC1100 were obtained. In addition, the deduced protein sequence encoded by the nucleotide sequence downstream of cleD on plasmid pAC27 of the 3-chlorobenzoate-degrading Pseudomonas putida AC866 was tested for homology. Significant sequence similarities with the polypeptides encoded by the tfdF, tcbF, and tfE genes as well as the nucleotide sequence downstream of the clcD gene gave evidence that these genes might encode maleylacetate reductases. A NAD-binding motif in a βαβ-element was detected.

The aerobic mineralization of chloroaromatic compounds appears to be governed by various pathways. For many chloroaromatic compounds such as chloroanilines, chlorobenzenes, chlorobenzoates, chlorobiphenyls, chlorophenols, and chlorophenoxyacetates, the degradative pathways involve various chlorosubstituted intermediates. A convergence at chlorosubstituted catechols as central intermediates was observed. The elimination of chloride takes place after cleavage of chlorocatechols. With other substrates the removal of chloro substituents occurs at an early stage of the degradative pathway with hydrolytic, oxygenolytic, or reductive dechlorination prior to ring cleavage. 1,2,4-Trihydroxybenzene with or without a chlorine substituent seems to be a common ring cleavage substrate in the degradation of pentachlorophenol, 2,4,5-trichlorophenoxyacetate, and 2,4,6-trichlorophenol (1, 7, 10).

Both types of pathways, the degradation of chloroaromatics via chlorocatechols and via 1,2,4-trihydroxybenzene, converge at the step of maleylacetate (Fig. 1). A maleylacetate reductase forms 3-oxoadipate, an intermediate shared with the degradation pathway of various aromatic compounds.

The first enzymes of the modified ortho pathway (Fig. 2a), chlororcatheol 1,2-dioxygenase, chloromuconate cycloisomerase, and dienelactone hydrolase, involved in chlorocatechol degradation, have been studied. They are encoded by degradative plasmids. Those which have been most thoroughly investigated are pJP4, encoding the 2,4-dichlorophenoxyacetate catabolic enzymes in Alcaligenes eutrophus JMP134 (8), pAC27, encoding the 3-chlorobenzoate degradative enzymes in Pseudomonas putida AC866 (4, 5), and pP51, encoding the enzymes for 1,2,4-trichlorobenzene catabolism in Pseudomonas sp. strain P51 (26). From Pseudomonas sp. strain B13 (9), the genes for chlorocatechol catabolism have been shown to be transmissible to various recipients (20). However, the plasmid, named pB13 or pWR1, was difficult to isolate and gave low yields of plasmid DNA (3). It was found to be very similar to pAC25, the precursor of pAC27 (2, 3). Furthermore, the dienelactone hydrolases purified from Pseudomonas sp. strain B13 and from a clone with pAC27 genes were found to be identical in every discernible respect (12, 18). The enzymes for chlorocatechol degradation of Pseudomonas sp. strain B13 and pAC27 seem to be, therefore, not different.

The genes encoding chlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase, and dienelactone hydrolase are located in operons of similar structures (20a) (Fig. 2b). Sequence analysis indicated a high degree of homology between the respective polypeptides (20a). While the functions of genes tfdCDE of pJP4, tcbCDE of pP51, and clcABD of pAC27 are known, no clear function has been attributed to the TfdF and TcbF proteins. The amino acid sequences predicted from the nucleotide sequences of tfdF and tcbF show 30% identity with alcohol dehydrogenases (22, 23). In contrast, the operon structure of the tf genes is totally different from that of the ortho pathway (7).

Maleylacetate reductases have recently been purified from Pseudomonas sp. strain B13, A. eutrophus JMP134, and 1,4-dichlorobenzene-grown cells of Pseudomonas aeruginosa RHO1 (13, 14, 17, 21). While the enzymes of strains B13 and RHO1 have the same size (they consist of two identical subunits of 37 kDa), the maleylacetate reductase of strain JMP134 was reported to be a dimer composed of two identical subunits of 35 kDa. Besides the function of reducing maleylacetate to give 3-oxoadipate, the enzymes showed dehalogenating activity for maleylacetates with halogen substituents in position 2 so that the convergence of the monochloro- and dichlorocatechol degradation is achieved (13, 15, 27).
First lines of evidence suggesting that \( \text{tfdF} \) and \( \text{tcbF} \) might encode a maleylacetate reductase were reported by Seibert et al. (21). The N-terminal part of the maleylacetate reductase from strain JMP134 shows some similarity (36.0% identity) to the N-terminal part of the predicted polypeptide encoded by the \( \text{tfdF} \) gene of the catabolic plasmid pJP4. The deduced protein sequence of the \( \text{tftE} \) gene of the 2,4,5-trichlorophenoxyacetate-degrading \textit{Burkholderia cepacia} AC1100 shows high percent identity to gene products of \( \text{tfdF} \) (52%) and \( \text{tcbF} \) (56%). It has been proven by substrate-enzyme assays that the polypeptide expressed by \( \text{tftE} \) in \textit{Escherichia coli} under the control of the T7 promoter is a maleylacetate reductase (7).

Here, we provide evidence that the \( \text{tfdF} \) and \( \text{tcbF} \) genes as well as the gene downstream of \( \text{clcD} \) encode maleylacetate reductases. This was done by an alignment of polypeptides originating from the purified maleylacetate reductase of strain B13 with the polypeptides predicted from the \( \text{tfdF} \) and \( \text{tcbF} \) genes. The alignment of these predicted polypeptides with the \( \text{tftE} \) gene product supports this statement.

The maleylacetate reductases from 3-chlorobenzoate-grown cells of \textit{Pseudomonas} sp. strain B13 and 1,4-dichlorobenzene-grown cells of \textit{P. aeruginosa} RHO1 were purified by a series of chromatographic steps (14, 17). The N-terminal amino acid sequences of the two enzymes were determined to be identical: MNFIHDYRSPRVIFGPDSLARLPQELERLGIDRALV. Comparison with the N-terminal sequence of the maleylacetate reductase of \textit{A. eutrophus} JMP134 showed limited identity (Fig. 3). A homology of only 33.3% was observed within the 27 amino acids. A comparison of the N-terminal amino acid sequences of the maleylacetate reductases from strain B13 and RHO1 with sequences in the Swiss Protein (Swiss-Prot) data bank revealed 58.3% homology with TcbF and 50.0% homology with TfdF. Alignment with TftE showed 52.8% identity.

The protein from strain B13 was digested by use of trypsin, so that oligopeptides flanked by lysine or arginine resulted. The protein (0.22 mg) was incubated for 16 h at 25°C with trypsin (25 \( \mu \)g/ml). The resulting mixture of oligopeptides was fractionated by high-performance liquid chromatography on an RP-18 column (Fig. 4). A gradient was obtained by mixing solvent A (0.1% trifluoroacetic acid in water) with solvent B (80% acetonitrile plus 0.085% trifluoroacetic acid in water). The following conditions were used in running the gradient: 0 to 5 min, 5% B; 5 to 90 min, 70% B; 90 to 95 min, 100% B; 95 to 100 min, 5% B. Fractions 1 to 20 were collected and lyophilized. The N-terminal sequences of 12 of the purified peptides were determined with an Applied Biosystems model 473A protein sequencer. The sequences were compared with sequences in the SwissProt database (as of December 1994) by using the FASTA program.

The alignment of these oligopeptides with the predicted polypeptides encoded by the \( \text{tfdF} \), \( \text{tcbF} \), and \( \text{tftE} \) genes is shown in Fig. 5. In addition, the peptide sequence encoded downstream of gene \( \text{clcD} \) is included after elimination of frameshifts, which led to disruption of the reading frame, by using the sequences obtained from the maleylacetate reductase of strain B13. The deduced amino acid sequence from plasmid pAC27 has 100% identity with the oligopeptides originated from strain B13 maleylacetate reductase, confirm-
ing the high homology of the proposed maleylacetate reductases. The oligopeptide sequences are up to 80% homologous to TcbF, TfdF, and TftE, a much higher homology than that reported for the maleylacetate reductase isolated from strain JMP134.

Since the maleylacetate reductases use NADH as a cofactor, we searched for a NAD-binding motif within the amino acid sequences. It is well known that NAD-binding sites exist at ββ-elements of proteins (28). Using the computer prediction (PC-Gene version 5.1, 1987; IntelliGenetics Inc.), we found a motif predicting a secondary structure with a ββ-element in the maleylacetate reductases approximately 90 amino acids from the N terminus. This structural element contains the essential parts of the NAD-binding fingerprint according to Wierenga et al. (28): BZxZxGxGxxGxxxZxxZx(x)3–6xZxZx(D/E). Amino acid B is either basic or hydrophilic, i.e., K, R, H, S, T, Q, N, or D, while Z is a small and hydrophobic amino acid, i.e., A, I, L, V, M, C, Y, G, F, or N. There are some differences between the sequences of the maleylacetate reductases and the typical consensus sequence predicted by Wierenga et al. (28). (i) The total length of the ββ-element of the enzyme from strain B13 or that encoded by plasmid pAC27 is 37 amino acids, in contrast to the 31 predicted. (ii) At the beginning of the ββ-element, one amino acid, glycine, is intercalated between the basic, hydrophobic asparagine at position 87 and the small, hydrophobic valine. (iii) In addition, one amino acid, isoleucine, threonine, or serine, ameliorates the GxGxxG structure of the enzymes encoded by pAC27, pP51, and pJP4 and the enzymes from the strains AC1100 and B13. (iv) There are some differences between the sequences of the maleylacetate reductases and the typical consensus sequence predicted by Wierenga et al. (28). (i) The total length of the ββ-element of the enzyme from strain B13 or that encoded by plasmid pAC27 is 37 amino acids, in contrast to the 31 predicted. (ii) At the beginning of the ββ-element, one amino acid, glycine, is intercalated between the basic, hydrophobic asparagine at position 87 and the small, hydrophobic valine. (iii) In addition, one amino acid, isoleucine, threonine, or serine, ameliorates the GxGxxG structure of the enzymes encoded by pAC27, pP51, and pJP4 and the enzymes from the strains AC1100 and B13. (iv) The loop between the α-helix and the second β-strand is larger in the enzymes discussed here. The comparison of the amino acid sequences of ClcE, TcbF, TfdF, and TtE as well as that of the enzyme from strain B13 illustrates that especially the region of the ββ-structure is highly conserved, confirming the functional identity of the enzymes induced for the degradation of different chlorocatechols and chlorotrihydroxybenzene.

FIG. 2. Enzyme sequence and operon structure of the genes of the modified ortho cleavage pathway with adjacent regulatory genes. (a) Modified ortho pathway for chlorocatechols including two dechlorinating steps in the degradation of di- and trichlorocatechols (Cl1 and Cl3). The ortho cleavage is indicated by a line of stars. (b) Each bar corresponds to a gene or an open reading frame (ORF). Homologous genes are represented by bars of the same type. The genes of the chlorocatechol 1,2-dioxigenases (clcA, tblC, and tbcC) are black, those of the chloromuconate cycloisomerases (clcB, tblD, tbcD) are dark grey, and those of the dienelactone hydrolases (clcD, tblE, and tcdE) are hatched. The genes tblF and tblG as well as the homologous pAC27 region (clcE) are light grey. The regulator genes clcR, tblR, and tbcR are medium grey. tblR is located several kilobases upstream of tblCDE. Arrows without tips denote an overlap of the reading frames. The information was compiled from references 6, 11, 16, 19, 24, 25.

FIG. 3. Comparison of the N-terminal amino acid sequence of the maleylacetate reductase (MAR) of strain B13 with those of strains JMP134 and RHO1 and the proteins encoded by tblF and tblG. Consensus amino acids are boldfaced.
The present data provide valuable evidence that the modified \textit{ortho} pathway genes \textit{tcbF}, \textit{tfdF}, and \textit{clcE} may encode maleylacetate reductases. However, some functional analyses of the gene products, such as enzyme assays, are essential before a definitive assignment of function can be made.

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