Construction of hybrid *xylE* genes between the two duplicate homologous genes from TOL plasmid pWW53: comparison of the kinetic properties of the gene products

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(Received 6 March 1990; revised 10 April 1990; accepted 25 April 1990)

The two *xylE* genes for catechol 2,3-oxygenase, encoded by TOL plasmid pWW53, carry a common *SaI*I restriction site within the reading frame. Each gene was cut at the *SaI*I site and the 5' end of each gene spliced to the 3' end of the other to form hybrid genes, from both of which catalytically active catechol 2,3-oxygenase activities were expressed. The kinetic parameters were determined for the gene products of both the hybrid and the wild-type *xylE* genes with catechol, 3-methylcatechol and 4-methylcatechol as substrates. Comparison of the results suggested firstly, that the C-terminal regions of the enzymes determined both the binding and the catalytic specificity, and, secondly, that the N-terminal region of one of the enzymic gene products contained a secondary binding site which caused inhibition by excess substrate for methylcatechol substrates but not for catechol. One of the wild-type enzymes appeared to have an intrinsically higher activity for all three substrates than the other. This higher activity depended on the presence of both its C- and N-terminal regions, and in both hybrid enzymes, which contained only one of these regions, activity was significantly reduced.

Introduction

Osborne *et al.* (1988) described the occurrence on TOL catabolic plasmid pWW53 of two separate operons for the *meta* pathway genes. These duplicate copies were homologous as judged by their ability to cross-hybridize and from a number of apparently similar restriction sites. Both copies were fully functional and could support growth on *m*-toluate when individually ligated into a vector plasmid and transferred into a TOL-plasmid-free strain of *Pseudomonas*. However there was a major difference in the level of expression of their encoded enzymes, the operon located on the smaller *Hind*III fragment HB producing only about 10 % of the specific activity produced by the operon found on *Hind*III fragment HA. The results could not distinguish whether this was the result of a difference in their regulatory mechanisms or due to intrinsically lower enzyme activities.

A comparison of the restriction maps for the regions carrying the two *xylE* genes (encoding catechol 2,3-oxygenase; C23O) showed that within the centre of each coding region there is an apparently common *SaI*I site (Osborne *et al.*, 1988). This paper describes the forma-

tion of two hybrid genes using this restriction site to ligate the 5' end of each to the 3' end of the other. The resulting genes both produced active gene products, showing that the two *SaI*I sites lie at identical locations within the genes. The kinetic properties of the two wild-type and two hybrid C23Os have been compared to ascertain whether any catalytic property can be assigned to either the N- or C-terminal regions of the proteins.

Methods

Bacterial strains and plasmids. Plasmids used and constructed in this work are shown in Table 1. The hosts for all experiments were *Escherichia coli* strains ED8654 or JM103.

Media and culture conditions. For enzyme assays, strains were grown on Luria broth and cell extracts prepared by sonication as described by Cane & Williams (1982).

Assay of catechol 2,3-oxygenase, and kinetic analysis. The activity of C23O was determined in cell extracts by the basic procedure of Sala-Trepat & Evans (1971). For the determination of kinetic constants, a range of about 15 concentrations of catechol and methylcatechol substrates from about 33 µM to about 2 mM were used in the standard assay mixture. The absolute concentrations of the stock solutions of substrates used to make up the individual reactions were determined by complete conversion to the ring-fission products by excess cell extract of *E. coli* strains carrying the cloned *xylE* gene on a high expression vector.

Abbreviation: C23O, catechol 2,3-oxygenase.

Table 1. *Plasmids*

Plasmid name	Notes
Vectors	
pFB69	Barany (1985)
pBR325	Prentki & Kirsch (1982)
pUC18	Yanisch-Perron <i>et al.</i> (1985)
pKT230	Bagdasarian <i>et al.</i> (1981)
Recombinant plasmids	
pWW53-3501	2.1 kbp <i>XhoI</i> fragment from pWW53 carrying wild-type Aa gene in pKT230 (Osborne <i>et al.</i> , 1988)
pWW53-3502	2.1 kbp <i>XhoI</i> fragment from pWW53 carrying wild-type Bb gene in pKT230 (Osborne <i>et al.</i> , 1988)
pWW53-3511	2.1 kbp <i>XhoI</i> fragment from pWW53 carrying Aa gene cloned into the <i>SaII</i> site of pUC18 (this study)
pWW53-3515	2.1 kbp <i>XhoI</i> fragment from pWW53 carrying Bb gene cloned into the <i>SaII</i> site of pUC18 (this study)
pWW53-5000	1.76 kbp partial <i>SaII</i> fragment from pWW53-3511 inserted into <i>XhoI</i> site of pKT230 (this study)
pWW53-5100	0.55 kbp <i>SaII</i> fragment from pWW53-3501 in pBR325 (this study)
pWW53-6000	1.76 kbp partial <i>SaII</i> fragment from pWW53-3515 inserted into <i>XhoI</i> site of pKT230 (this study)
pWW53-6100	0.55 kbp <i>SaII</i> fragment from pWW53-3502 in pBR325 (this study)
pWW53-5500	0.65 kbp <i>SaII/XhoI</i> fragment of pFB69 inserted into the unique <i>SaII</i> site of pWW53-5000 (this study)
pWW53-6500	0.65 kbp <i>SaII/XhoI</i> fragment of pFB69 inserted into the unique <i>SaII</i> site of pWW53-6000 (this study)
pWW53-5600	0.55 kbp <i>SaII</i> fragment of pWW53-6100 inserted into the unique <i>SaII</i> site of pWW53-5500 to form the hybrid Ab gene (this study)
pWW53-6600	0.55 kbp <i>SaII</i> fragment of pWW53-5100 inserted into the unique <i>SaII</i> site of pWW53-6500 to form the hybrid Ba gene (this study)

The amounts of cell extract added were chosen to ensure that the rate of change of absorbance due to production of ring-fission product was linear over 1 min and no greater than 1.0 min^{-1} . The rate of reaction was determined using the microcomputer regression analysis afforded by the Kontron spectrophotometer, based upon the first 10 absorbance measurements made at 3 s intervals. The molar absorption coefficients for the three products were taken as ($\text{M}^{-1} \text{ cm}^{-1}$): catechol, 36×10^3 at $\lambda_{\text{max}} = 375 \text{ nm}$; 3-methylcatechol, 15×10^3 at $\lambda_{\text{max}} = 388 \text{ nm}$; and 4-methylcatechol, 31.5×10^3 at $\lambda_{\text{max}} = 382 \text{ nm}$.

The values of K_m and V were calculated by non-linear regression analysis according to Wilkinson (1961) using the computer program ENZPACK3 (Williams & Zaba, 1989). Because of substrate inhibition discovered during the course of this work, only substrate concentrations below which the effect was not apparent ($\leq 500 \mu\text{M}$) were used in the calculation of the parameters; this was done even for enzyme/substrate combinations where there appeared to be no substrate inhibition. Routinely seven or eight rate measurements in this range were used to calculate K_m and V . For each enzyme/substrate combination, five or six separate determinations of the parameters were carried out, and the values quoted are the means and standard deviations of all of these results.

Plasmid and DNA manipulations. All experiments were carried out according to procedures outlined by Osborne *et al.* (1988).

Results and Discussion

Construction of hybrid genes

The two *xylE* genes were initially available on 2.1 kbp *XhoI* fragments cloned into the *XhoI* site in pKT230.

Recombinant plasmid pWW53-3501 (Fig. 1a) carried the gene from the HA fragment of pWW53; the restriction map of its insert indicates that the gene corresponds to type 1a, as denoted by Chatfield & Williams (1986). For convenience we will refer to this gene (and its enzyme product where applicable) as type Aa, denoting its 5' end up to the *SaII* site, by the capital A and the 3' end from the *SaII* site, by the lower case a. Plasmid pWW53-3502 carries the gene from the HB fragment of pWW53, which corresponds to type 1b (Chatfield & Williams, 1986) and we shall refer to this as type Bb, using the same notation. Initially, each of the *XhoI* inserts of pWW53-3501 and pWW53-3502 was excised and religated into the *SaII* site of pUC18 to form plasmids pWW53-3511 and pWW53-3515 respectively. The hybrid *SaII/XhoI* sites formed at each terminus of the inserts can no longer be cut with either restriction enzyme.

Since the principles of constructing the two hybrid genes are essentially identical, we shall describe only the construction of the gene between the 5' end of the Aa gene and the 3' end of the Bb gene, forming the Ab gene. The equivalent plasmids in the formation of the Ba hybrid are included in Table 1.

Each *XhoI* insert has three *SaII* sites, one within the *xylE* gene and one at either end (Fig. 1a). In order to obtain a fragment with a unique *SaII* site within the gene, plasmid pWW53-3511 was partially digested with *SaII*

Fig. 1. Accurate restriction maps of the inserts carrying *xylE* genes. (a) Wild-type gene of type Aa on 2.1 kbp *XhoI* fragment in plasmids pWW53-3501 and pWW53-3511. (b) Wild-type gene of type Bb on 2.1 kbp fragment in plasmids pWW53-3502 and pWW53-3515. (Note that in pWW53-3511 and pWW53-3515 the sites at the end of the insert are X/S rather than X.) (c) Hybrid gene of Ab type on plasmid pWW53-5600. (d) Hybrid gene of type Ba on plasmid pWW53-6600. The inserts are denoted by the thin lines and the termini of the vector plasmids by the thick lines. The location of the *xylE* genes and their respective domains are shown above each map. The abbreviations for restriction sites are: A, *AvaI*; B, *BamHI*; Bs, *BstEII*; P, *PstI*; Pv, *PvuII*; S, *SaII*; X/S, hybrid *XhoI/SaII* site formed by inserting a *SaII* fragment into an *XhoI* site; Sm, *SmaI*; Ss, *SstI*; X, *XhoI*; Xb, *XbaI*.

and recloned into the *XhoI* site of pKT230 to form hybrid *XhoI/SaII* termini (Fig. 2). After transformation, the plates were sprayed with catechol to detect strains which had acquired a functional C23O and therefore carried the intact Aa gene with the internal *SaII* site unaltered. The resulting plasmid from such a transformant was retained as pWW53-5000, after checking that it had the expected restriction map.

The 3' end of the Bb gene was isolated by digestion of pWW53-3502 with *SaII* and ligation into the *SaII* site of pBR325, forming plasmid pWW53-6100. The strategy was then to introduce the insert from this plasmid into the (now) single *SaII* site of plasmid pWW53-5000. Since this was expected to generate a functional hybrid gene, if inserted in the correct orientation, there was no selection which would enable us to distinguish between transformants which contained recircularized pWW53-5000, and therefore a functional Aa gene, and those with a functional hybrid Ab gene: both would produce yellow transformants after spraying with catechol.

To overcome this problem, we inserted a neutral piece of DNA into the *SaII* site of pWW53-5000 to inactivate the *xylE* gene. The fragment arbitrarily chosen for this was a 0.65 kbp *SaII-XhoI* fragment from plasmid pFB69. Triple *SaII/XhoI/PstI* digestion of pFB69 produces three fragments, one of which is the desired neutral piece of 0.65 kbp. The triply-digested pFB69 was ligated with *SaII*-digested pWW53-5000 and transformants were selected which did not go yellow on spraying with catechol. Since the insert could be in two orientations, resulting recombinants were screened to ensure that the single *SaII* site remaining was at the 3' end of the 5' section (A) of the gene, as in the retained plasmid pWW53-5500 (Fig. 2).

The final stage was to insert the *SaII* fragment from pWW53-6100, carrying the 3' end of the Bb gene, into the single *SaII* site of pWW53-5500, selecting for C23O-positive strains, which contained the hybrid Ab gene. The resulting plasmid, pWW53-5600 (Fig. 2) was checked for the expected configuration of restriction sites (Fig. 1c).

Plasmid pWW53-6600, carrying the hybrid Ba gene (Fig. 1d) was constructed in an exactly analogous way, starting with plasmids pWW53-3515 (Fig. 1b) and pWW53-3501 (Fig. 1a).

Enzyme activity of gene products

Using the standard assay procedure for C23O (Sala-Trepat & Evans, 1971), in which the substrate concentrations are 0.1 mM, cell extracts from *E. coli* strains carrying the wild-type and hybrid genes all contained significant activity against catechol, 3-methylcatechol and 4-methylcatechol (data not shown). The specific activity due to the Bb gene product was considerably lower than that due to the Aa gene product, as had been reported earlier (Osborne *et al.*, 1988). In the strains carrying the Ab and Ba genes the specific activities were low and comparable to that carrying the Bb gene. The absolute activities and the relative values for the three substrates are of no significance in the light of the features revealed in the subsequent kinetic analysis.

Kinetics of gene products

Detailed kinetic analysis of the four C23O enzymes in cell extracts of *E. coli* indicated a feature not previously reported for any ring-cleavage dioxygenase. Two of the enzymes (Aa and Ab) showed significant classical substrate inhibition with the two methylcatechols at concentrations above 0.5 mM but no such effect with catechol itself. A typical set of Michaelis-Menten curves is shown for enzyme Aa in Fig. 3(a). The other two

Fig. 2. Formation of a plasmid carrying a hybrid *xylE* gene of type Ab. The relevant abbreviations for restriction enzymes are as in Fig. 1. The sizes of the plasmid circles (without their shown insert) are indicated inside the circles. The location of the *xyl* genes and domains thereof are shown beside the DNA in a bold line. Relevant plasmid genes are shown in their rough location: Ap^r, ampicillin resistance; Sm^r, streptomycin resistance; *ori*, replicative origin. The maps are not drawn to scale: for accurate sizes of the relevant DNA fragments see Fig. 1.

enzymes (Bb and Ba) showed no such effect with catechol or with 4-methylcatechol and showed a marginal effect with 3-methylcatechol (see Fig. 3*b*). If high concentrations of 3-methylcatechol are inhibitory with these enzymes, then the effect is much weaker than with the Aa and Ab proteins. Since the common element of the two enzymes which show this effect is the N-terminal domain from the Aa protein, these results suggest that it is this region which is responsible for this effect, presumably because of its capacity for secondary substrate binding.

Because of this substrate inhibition, only the seven or eight values where $[S] \leq 0.5$ mM were used to determine K_m and V . The values quoted in Table 2 are derived from five or six separate determinations using fresh cell extracts on each occasion.

The results are presented in graphical form in Fig. 4. Comparison of the K_m values for the three substrates and the four enzymes shows that the specificity profiles of the Aa and the Ba enzymes are similar, with highest K_m values for catechol, whereas the Bb and the Ab enzymes have a different profile with highest K_m values for 4-methylcatechol (Fig. 4*a*). If the K_m is taken as a measure of the affinity of the protein for the substrate, this would lead to the conclusion that the site on the proteins determining their substrate affinity is located on their C-terminal halves, and that the 'a' domain had a weak affinity for catechol and the 'b' domain a weak affinity for 4-methylcatechol.

The values of V are a measure of the catalytic activity of the extracts. Differences in the values could be the result of differences either in the intrinsic activity of

Fig. 3. Typical Michaelis-Menten curves for wild-type C23Os acting on catechol and the methylcatechols: (a) enzyme of type Aa; (b) enzyme of type Bb. The curves for enzyme of type Ab are similar to those for Aa, as are those for type Ba to type Bb.

the two gene products or in the levels of transcription from the vector promoters due to the different intervening DNA in the recombinant plasmids. In the plasmids pWW53-3501 and pWW53-5600, this intervening DNA is not identical but differs by the deletion of a 0.23 kbp *XhoI*-*SalI* fragment in the latter (cf. Figs 1a and 1c). It is possible, but unlikely, that the level of transcription is depressed in the latter as a direct result of a deletion bringing the promoter closer to the structural gene. It is more likely that any significant difference in V is the result of a difference in the intrinsic catalytic activity of the gene product. The same argument can be applied to pWW53-3502 and pWW53-6600 (Figs 1b and 1d). The values of V therefore show that the introduction of the C-terminal end of the wild-type Bb protein reduces the catalytic activity of the Aa protein. Conversely the

introduction of the C-terminus of the Aa protein into the Bb protein does not enhance its activity significantly. Thus either half of the Bb protein depresses the V , which we assume to be due to its intrinsically lower catalytic potential. Although not as clearly as in the case of the K_m values comparison of the specificity profiles for V (Fig. 4b) suggests some linkage between the domains and the catalytic specificity. In fact the substrate profiles for both K_m and V for all the enzymes look very similar (Fig. 4a, b), with a relatively high K_m matched by a high V . Thus for example the Aa protein appears to bind catechol less strongly than the methylcatechols (high K_m) but catalyses its breakdown more effectively (high V). This is mirrored, but at lower levels, by the Ba protein, suggesting that the catalytic determinants are also concentrated in the C-terminal regions. The compensa-

Fig. 4. Kinetic parameters calculated for wild-type and constructed hybrid C23Os plotted from Table 2. (a) Michaelis constant (K_m) values; (b) maximum velocity (V) values. $\square$, Catechol; $\blacksquare$, 3-methylcatechol; $\hatched$, 4-methylcatechol.

tory effect of high K_m and high V can be seen when the 'specificity constants' (V/K_m) are compared for all four proteins (Table 2). Although the values differ significantly between Aa and the other enzymes (reflecting its greater catalytic effectiveness), the values for the three substrates for each protein are roughly the same.

In previous papers the substrate specificities of catechol 2,3-oxygenases have been compared using the reaction rates determined in the standard assay procedure (see Keil *et al.*, 1985). The kinetic analysis shows clearly that this can be a flawed procedure. The concentrations of substrates used in the assay (≈ 0.1 mM; Sala-Trepat & Evans, 1971) are in most cases below the K_m value, and therefore the measured velocities do not approach the V values: ideally the concentrations should be $\geq 5 \times K_m$. As a result of this investigation we have increased the concentration of catechols in our standard assay procedure to 1 mM. However this also has its dangers because of the possibility of substrate inhibition at this higher concentration. The only certain procedure to assess substrate specificities is to analyse the kinetics over a range of substrate concentrations and determine the kinetic parameters.

The position of the *Sa/I* site at which the fusion of the two genes has been effected has not yet been determined by DNA sequencing, but the *xyIE* gene from pWW0 has been sequenced (Nakai *et al.*, 1983) and this has a configuration of restriction sites very similar, but not

Table 2. Kinetic parameters for wild-type and hybrid catechol 2,3-oxygenases

K_m measured in mM; V measured in milliunits $(mg \text{ protein})^{-1}$. Values quoted are the mean $\pm$ SD for five or six independent determinations with fresh cell extracts.

Enzyme type	Parameter	Substrate		
		Catechol	3-Methylcatechol	4-Methylcatechol
Aa	K_m	0.290 ± 0.040	0.135 ± 0.021	0.201 ± 0.036
	V	1400 ± 160	620 ± 100	1010 ± 150
	V/K_m	4800	4600	5000
	Substrate inhibition	—	+	+
Bb	K_m	0.218 ± 0.036	0.208 ± 0.034	0.518 ± 0.14
	V	350 ± 40	185 ± 16	530 ± 80
	V/K_m	1600	890	1000
	Substrate inhibition	—	$\pm$	—
Ab	K_m	0.095 ± 0.015	0.113 ± 0.020	0.252 ± 0.069
	V	161 ± 11	157 ± 12	380 ± 60
	V/K_m	1700	1400	1500
	Substrate inhibition	—	+	+
Ba	K_m	0.494 ± 0.036	0.297 ± 0.058	0.294 ± 0.078
	V	640 ± 110	280 ± 60	300 ± 80
	V/K_m	1300	930	1000
	Substrate inhibition	—	$\pm$	—

identical, to the Aa gene (see Chatfield & Williams, 1986). In the pWW0 gene the *SalI* site spans bases 579–584, coding for amino acids Leu-Ser-Thr (193–195) in the sequence of 306 amino acid residues. Assuming that the *SalI* site is in the same location in the Aa and Bb genes, the N-terminal region covers the first 194 residues and the C-terminal region the final 112 residues.

The method used in this paper of constructing *in vitro* hybrids between homologous genes clearly has limitations in its general applicability as a method of assigning functions to relevant regions of their gene products. Not the least of these is whether particular biochemical properties of a protein (catalysis, binding, pH variations, subunit association, etc.) can be definitively assigned to a unique region of the linear sequence. However, within these limitations of interpretation, the results presented here indicate that: (a) the two C23O enzymes from pWW53 differ significantly in their intrinsic catalytic activity against all three substrates tested; (b) the C-terminal region of each appears to be dominant in determining their substrate specificity, with regard to both binding strength and catalysis, which would further suggest that the main active-site amino acids were located in this region; (c) the N-terminal region of the Aa protein carries a site which makes the enzyme susceptible to inhibition by excess concentrations of the two methylcatechols.

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