Molecular characterization of PadA, a phenylacetaldehyde dehydrogenase from *Escherichia coli*

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Abstract The *padA* gene encoding the phenylacetaldehyde dehydrogenase involved in the catabolism of 2-phenylethylamine in *Escherichia coli* has been cloned, sequenced, and located at 31.0 min on the chromosome. The deduced PadA polypeptide contains 499 amino acid residues with a predicted molecular mass of 53.7 kDa, and its primary structure reveals significant similarity with that of members of the aldehyde dehydrogenase superfamily. By engineering optimal transcription and translation elements, a high expression of the *padA* gene has been achieved. The active PadA enzyme is a homodimer that prefers NAD^+ over $NADP^+$ as coenzyme. The enzyme efficiently oxidizes only phenylacetaldehyde-like aromatic aldehydes, and has a weak esterase activity with *p*-nitrophenol. The *padA* gene constitutes a new catabolic tool for designing DNA cassettes to expand the abilities of microorganisms to degrade toxic aromatic compounds.

Key words: Phenylacetaldehyde dehydrogenase; Gene expression; *Escherichia coli*; Catabolism; Primary structure

1. Introduction

Phenylacetaldehyde (PAL) is an aromatic compound that in microorganisms is produced by (i) decarboxylation of phenylpyruvic acid during the catabolism of L-phenylalanine [1], (ii) oxidative deamination of biogenic amines such as 2-phenylethylamine [2,3], and (iii) isomerization of styrene oxide or oxidation of 2-phenylethanol during the catabolism of some toxic aromatic compounds such as styrene and ethylbenzene [4,5]. Growth of *Escherichia coli* K-12 on 2-phenylethylamine as the sole carbon and energy source induces two enzymatic activities, an amine oxidase that converts 2-phenylethylamine into PAL, and a phenylacetaldehyde dehydrogenase that oxidizes the latter to phenylacetic acid (PA) [2] (Fig. 1). The *maoA* gene encoding the 2-phenylethylamine oxidase (MaoA) from *E. coli* has been cloned, sequenced, overexpressed [6], and its regulation described [7]. Moreover, the purification, characterization and crystallization of MaoA have also been reported [6]. In contrast, the molecular characterization of the gene encoding the phenylacetaldehyde dehydrogenase of *E. coli* remains to be done. Surprisingly, there are also no reports on the characterization of the genes encoding other phenylacetaldehyde dehydrogenases such as those acting on the processes mentioned above.

Here we report the cloning, sequencing, chromosomal localization, and overexpression of the gene encoding the phenylacetaldehyde dehydrogenase from *E. coli*. Some kinetic and structural properties of the enzyme have also been studied.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The bacterial strains used were *E. coli* W ATCC11105 [8], *E. coli* W14 (this work), and *E. coli* K-12 strain DH5α [9]. Plasmids pBR322 [9], pUC18 [9], and pIN-III(lpp^p-5)-A3 [10] were used in this study. Unless otherwise stated, bacteria were grown in Luria-Bertani (LB) medium [9] at 37°C with vigorous shaking. When required, M63 minimal medium [11] was used. Media were solidified with 1.5% Bacto agar (Difco Laboratories, Detroit, MI). Where appropriate, ampicillin (100 μg/ml) was added.

2.2. Isolation of E. coli W14

To isolate *E. coli* W mutants deficient in the catabolism of PA, the wild-type W strain was cultivated at 30°C on M63 minimal medium containing vitamin B_{12} (5 ng/ml) and PA as the sole carbon source, until the cultures reached an optical density at 600 nm of 0.8. Then, about 3.6×10^3 cells were plated onto minimal medium plates containing vitamin B_{12}, 5 mM 3-fluor-phenylacetic acid (3-F-PA), and 10 mM glycerol as carbon source. Since 3-F-PA is toxic for cells able to catabolize PA, surviving colonies should be PA^-. Several colonies were obtained and one of them, named *E. coli* W14, was checked for its inability to grow on minimal medium containing 5 mM PA as the sole carbon source.

2.3. DNA manipulations and sequencing

DNA manipulations and other molecular biology techniques were essentially as described [9]. DNA fragments were purified using low-melting point agarose. Southern blot analyses were performed as previously reported [9], using, as probe, DNA fragments labelled by the random primer method [9] with [α-^{32}P]dCTP. Plasmid DNA was prepared by the rapid alkaline lysis method, and cells were transformed through the RbCl procedure [9]. Nucleotide sequences were determined directly from plasmids using the dideoxy chain termination method [12]. Standard protocols of the manufacturer for *Taq* DNA polymerase-initiated cycle sequencing reactions with fluorescently labelled dideoxynucleotide terminators (Applied Biosystems Inc.) were used. The sequencing reactions were analyzed using a 377 automated DNA sequencer (Applied Biosystem Inc.). Sequences were extended by designing primers based on the already determined sequence. Protein sequence similarity searches and sequence alignments were made at the Baylor College of Medicine-Human Genome Center server.

2.4. Isolation of the padA gene

The *padA* gene was PCR-amplified from plasmid pFA2 using primers PadA-5 5′-GGTCTAGA**TTAACTTTA**G*TAAGGAGGT*AAAGTGatgACAGAGCCGCATGTAG-3′ and PadA-3 5′-CCGGATCCAACGCTAGGGGCGCTGAAG-3′ (engineered *Xba*I and *Bam*HI restriction sites are underlined and double-underlined, respectively; and the ideal Shine-Dalgarno region [13] and the translational enhancer sequence from gene 10 of phage T7 [14] are indicated in italic and bold-face letters, respectively; lowercase letters show the ATG initiation codon). The PCR product was cleaved with the appropriate restriction enzymes and gel-purified prior to its ligation to the vector.

2.5. Preparation of crude extracts and enzyme assays

Cells were cultured overnight at 30°C in LB medium in the presence

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Abbreviations: PAL, phenylacetaldehyde; PA, phenylacetic acid

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of 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) or 5.0 mM 2-phenylethylamine, when required. Cells were harvested by centrifugation, washed and resuspended in 0.1 volume of 100 mM potassium phosphate buffer, pH 8.0, prior to disruption by passage through a French press (Aminco Corp.) operated at a pressure of 20 000 psi. The cell debris was removed by centrifugation at 18 000 rpm for 30 min in a SS-34 rotor (Sorvall Inst.). The clear supernatant fluid was carefully decanted and used as crude extract. Protein concentration was determined by the method of Bradford [15] using bovine serum albumin as standard.

Standard assays of PadA activity in crude extracts were performed at 30°C in 100 mM potassium phosphate buffer, pH 8.0, with 10 μM PAL and 750 μM NAD^+ as substrates. The reactions were followed by monitoring the increase in absorbance at 340 nm and a molar absorption coefficient of 6220 M^{-1} cm^{-1} was used to determine the amount of NADH formed. To examine the substrate specificity of PadA, we used 750 μM NAD^+ and 10 μM of the aldehyde substrates.

The ability of PadA to hydrolyze *p*-nitrophenyl acetate was determined by measuring the increase in absorbance at 400 nm of the released *p*-nitrophenol (molar absorption coefficient of 98 000 M^{-1} cm^{-1}). The reaction was carried out at 30°C in 100 mM potassium phosphate buffer, pH 8.0, with 50 μM of *p*-nitrophenyl acetate. The rate of the spontaneous hydrolysis of *p*-nitrophenyl acetate was subtracted from the observed rate, and it was shown to be similar to the rate obtained with a crude extract from *E. coli* DH5α (pIN-III(lpp^p-5)-A3) cells.

2.6. Analytical methods

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 8% polyacrylamide slab gels as described [16]. Proteins were stained with Coomassie brilliant blue R250. The amino-terminal sequence of PadA was determined by Edman degradation with a 477A automated protein sequencer (Applied Biosystem Inc.). The protein was directly electroblotted from a SDS-polyacrylamide gel onto a polyvinylidene difluoride membrane as previously described [17]. Analytical gel filtration for determination of the molecular mass of active PadA was carried out on a Pharmacia Superose-12 FPLC gel filtration column, using 100 μl of a crude extract from *E. coli* DH5α (pAFC1) and a mobile phase of 100 mM potassium phosphate buffer, pH 7.0, at a flow rate of 0.3 ml per min. Absorbance of the effluent was monitored at 280 nm and PadA activity was checked. The molecular mass of PadA was determined from the relative mobility compared to that of the standard proteins (molecular mass values in parentheses), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), serum albumin (67 kDa), alcohol dehydrogenase (150 kDa), and catalase (240 kDa).

3. Results and discussion

3.1. Cloning, sequencing and chromosomal location of the padA gene

We have isolated an *E. coli* W mutant, strain W14, unable to grow on PA as the sole carbon and energy source (see Section 2). 2-Phenylethylamine was also unable to support growth of the W14 mutant, a result that is in agreement with the previous observation that 2-phenylethylamine catabolism in *E. coli* produces PAL which is subsequently oxidized to PA [2] (Fig. 1). Interestingly, *E. coli* W14 did not show any of the two enzymatic activities present in the wild-type strain that are required for the conversion of 2-phenylethylamine to PA, i.e. 2-phenylethylamine oxidase (data not shown) and phenylacetaldehyde dehydrogenase (Table 1). Hence, these results indicated that *E. coli* W14 was defective not only in PA degradation but also in the transformation of 2-phenylethylamine into this aromatic acid.

A *Bam*HI DNA library of the wild-type W strain was constructed using plasmid pBR322, and we were able to isolate a recombinant plasmid, pFA2, containing a 33.3-kb DNA insert (Fig. 2A), that conferred on *E. coli* W14 the ability to grow on either PA or 2-phenylethylamine as the sole carbon source. Furthermore, cellular extracts of *E. coli* W14 (pFA2) showed phenylethylamine oxidase (data not shown) and phenylacetaldehyde dehydrogenase (Table 1) activities, indicating that the genes encoding these enzymes were present in the 33.3-kb DNA fragment of plasmid pFA2. To identify the gene encoding the phenylacetaldehyde dehydrogenase of *E. coli* W, a *Hin*dIII library of the 33.3-kb DNA insert of plasmid pFA2 was constructed on the pUC18 cloning vector, and several of the resulting plasmids were sequenced. A 0.9-kb *Hin*dIII fragment (Fig. 2A) showed that 373 bp of one of its ends were nearly identical to those of the 3′-end of the *maoA* gene (accession number D23670) which has been mapped at 31.0 min on the *E. coli* chromosome [3]. Analysis of the remaining sequence of this *Hin*dIII fragment revealed the existence of a truncated open reading frame (ORF) encoding a product that presented a significant similarity to the COOH-terminus of aldehyde dehydrogenases. The complete sequence of this ORF was determined by using synthetic primers and plasmid pFA2 as source of DNA. Fig. 2B shows the sequence of a 2397-bp DNA fragment that carries: (i) the 3′-end of the *maoA* gene, (ii) the 5′-end of the *maoB* gene, encoding the transcriptional activator of *maoA* [7], and (iii) a 1500-bp ORF, *padA* gene, located between the genes *maoA* and *maoB*. The putative ATG initiation codon of *padA* is located at position 549 of Fig. 2B and is preceded by a consensus Shine-Dalgarno sequence (TAAGGA) [13]. The *padA* promoter region contains potential −10 and −35 sequences (Fig. 2B) that show significant similarity to those found in *E. coli* σ^{70}-dependent promoters [13]. Upstream of the −35 hexamer there is a $(A)_6$ tract (position 483–488) that could behave as the upstream (UP) element that stimulates transcription in certain promoters [18]. A palindromic sequence ($\Delta G = -9.1$ kcal/mol) followed by a $(T)_7$ tract is located 24 bp downstream of the TAA stop codon of *padA* (Fig. 2B), and may act as a ρ-independent transcription terminator. The *padA* gene exhibits a G+C content (53.4%) typical of *E. coli* genes, and analysis of its codon usage predicts that this gene encodes a non-abundant protein species [19].

Table 1
Phenylacetaldehyde dehydrogenase in different *E. coli* strains

E. coli strain	0.2 mM IPTG	Specific phenylacetaldehyde dehydrogenase activity (μmol min^{-1} per mg protein)
W	−	0.05
W14	−	b.d.
W14 (pFA2)	−	0.12
DH5α (pIN-III(lpp^p-5)-A3)	+	b.d.
DH5α (pAFC1)	−	1.03
DH5α (pAFC1)	+	2.66

Cells were grown overnight at 30°C in LB medium with (W and its derivatives) or without (DH5α derivatives) 5 mM 2-phenylethylamine. IPTG was added when indicated. Preparation of cellular extracts and determination of phenylacetaldehyde dehydrogenase activity were as described in Section 2. Results of one experiment are given; values were reproducible in three separate experiments. b.d. = below detection limits.

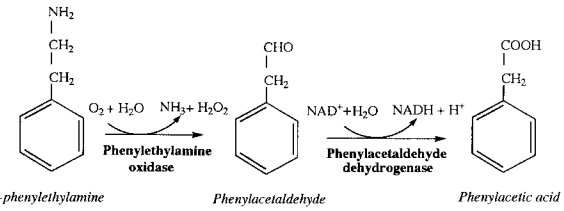

Fig. 1. Initial steps in the catabolism of 2-phenylethylamine by *E. coli*.

The *padA* gene product, PadA protein, contains 499 amino acid residues with a predicted molecular weight of 53.7 kDa and a deduced isoelectric point of 5.35. Comparisons of the deduced amino acid sequence of PadA with protein sequences available from the databases revealed the greatest sequence identities with several aldehyde dehydrogenases such as the human cytosolic aldehyde dehydrogenase (37.7%), *E. coli* betaine-aldehyde dehydrogenase (37.0%), *Pseudomonas putida* 2-hydroxymuconic semialdehyde dehydrogenase (35.8%), mouse mitochondrial precursor aldehyde dehydrogenase (35.6%), *E. coli* 5-carboxymethyl-2-hydroxymuconic semialdehyde dehydrogenase (35.0%), and *P. putida* benzaldehyde dehydrogenase (32.2%) [20–22]. Especially remarkable is the conservation in the PadA primary structure of residues Glu-272 and Cys-306, since the corresponding Glu-268 and Cys-302 residues in class 1 and 2 mammalian aldehyde dehydrogenases have been shown to be involved in the active site of these enzymes [20,22]. In this sense, the PadA sequence 297-GSFLNQG-QVCAA-308 matches perfectly the (SAG)XFXXXGQXC-X(AGN) aldehyde dehydrogenase active site motif (Fig. 2B). The aldehyde dehydrogenase consensus motif, (FY)(TV)G(S-NE)(TNSP)XX(GF), which may be involved in coenzyme (NAD^+ or $NADP^+$) binding [20], is also found in PadA (248-FTGSTATG-255). Moreover, residue His-235, which is essential for the dehydrogenase activity of cytosolic mammalian aldehyde dehydrogenases [23], is also conserved (His-240) in PadA (Fig. 2B).

In summary, amino acid sequence comparison together with the location of *padA* in the vicinity of the *maoA* and *maoB* genes, involved in the catabolism of phenylethylamine, suggest that PadA is the phenylacetaldehyde dehydrogenase of *E. coli* W and a new member of the aldehyde dehydrogenase superfamily. Interestingly, the *padA* gene is transcribed in the opposite direction to that of *maoA* and *maoB* (Fig. 2A), indicating that these three genes, although involved in the same metabolic pathway and physically associated at 31.0 min on the chromosome, do not constitute an operon.

3.2. Overexpression of the padA gene and characterization of its product

The *padA* gene has been PCR-amplified and cloned into the expression vector pIN-III(lpp^p-5)-A3 under the control of both a modified strong lipoprotein promoter and the *lac* promoter operator (Fig. 2A). To optimize translation, an ideal Shine-Dalgarno region and a translation enhancer sequence were engineered at an optimal distance from the ATG initiation codon of *padA* (see Section 2). SDS-PAGE analysis of crude lysates from *E. coli* DH5α cells containing the resulting plasmid pAFC1 showed the presence of an intense band corresponding to a protein with an apparent molecular weight of 55 kDa (Fig. 3), in good agreement with the predicted M_r for the PadA protein. The addition of 0.2 mM IPTG to the culture medium of cells containing plasmid pAFC1 increased the production of the 55 kDa protein (Fig. 3). The amino-terminal sequence, (M)TEPHVAVLS, of the overproduced protein was in complete agreement with that deduced from the nucleotide sequence of the *padA* gene, thus confirming this protein as the *padA* gene product and showing that no processing of its amino-terminal end occurs. As already reported for other overproduced recombinant proteins [24], cleavage of the initial Met was not complete and about 35% of the PadA polypeptide chains retained this amino acid. Analysis of the dehydrogenase activity present in extracts of IPTG-induced *E. coli* DH5α (pAFC1) cells revealed an increase by 53-fold over the activity detected in extracts of phenylethylamine-induced *E. coli* W (Table 1).

The PadA protein was partially purified from a crude extract of *E. coli* DH5α (pAFC1) by means of FPLC gel filtration chromatography. A major protein band corresponding to the PadA polypeptide chain was observed on SDS-PAGE (Fig. 3). Since the estimated molecular mass of the active PadA protein was 115 000 kDa (data not shown), this enzyme appears to consist of two identical subunits. Although many of the aldehyde dehydrogenases so far characterized are homotetramers, the quaternary structure in this superfamily of enzymes is evolutionarily variable and several homodimers have been also described [20–22].

The maximum reaction rate for PAL oxidation at saturating NAD^+ concentration (750 μM) was achieved with 6 μM PAL. In agreement with previous observations in *E. coli* K-12 [2] and *Achromobacter eurydice* [1] where a high concentration of PAL inhibited the phenylacetaldehyde dehydrogenase activity, we found that 40 μM of PAL at saturating NAD^+ concentration reduced by 50% the maximum reaction rate. Substrate inhibition was also observed in other aldehyde dehydrogenases [21,25]. The stoichiometry of the reaction between NAD^+ and PAL was determined to be 1:1. The highest reaction rate of PadA for NAD^+ reduction at saturating PAL concentration (10 μM) was obtained with 300 μM NAD^+. Under identical conditions, the maximum reaction rate was observed at a concentration of $NADP^+$ over 16-fold higher than that of NAD^+, thus suggesting that PadA is a NAD^+-dependent enzyme. This result is in agreement with the observation that most aldehyde dehydrogenases from both prokaryotic and eukaryotic organisms are NAD^+-specific [1,20,22], even though $NADP^+$-dependent phenylacetaldehyde dehydrogenases have also been reported [4,5]. Although the affinity of PadA for NAD^+ was two orders of magnitude lower than that for PAL, this may be compensated for by the high concentration (806 μM) of the NAD^+/NADH pool in *E. coli* [13]. On the other hand, since a poor expression of the *padA* gene can be predicted (see above), the high affinity of PadA for PAL might constitute an effective safety mechanism to eliminate free aldehyde that could be toxic to the cell.

In contrast to the phenylacetaldehyde dehydrogenase from *A. eurydice* [1], the PadA enzyme did not show activation by univalent cations (K^+ and Na^+) at concentrations ranging from 2 to 100 mM in 50 mM Tris-HCl buffer, pH 8.0. Enzyme assays of PadA in different buffers at different pHs revealed that the activity increased with increasing pH over the range examined, i.e. maximum activity was observed at pH 8.2 using 100 mM potassium phosphate buffer and at pH 8.9 using 50 mM Tris-HCl buffer (data not shown). Hence,

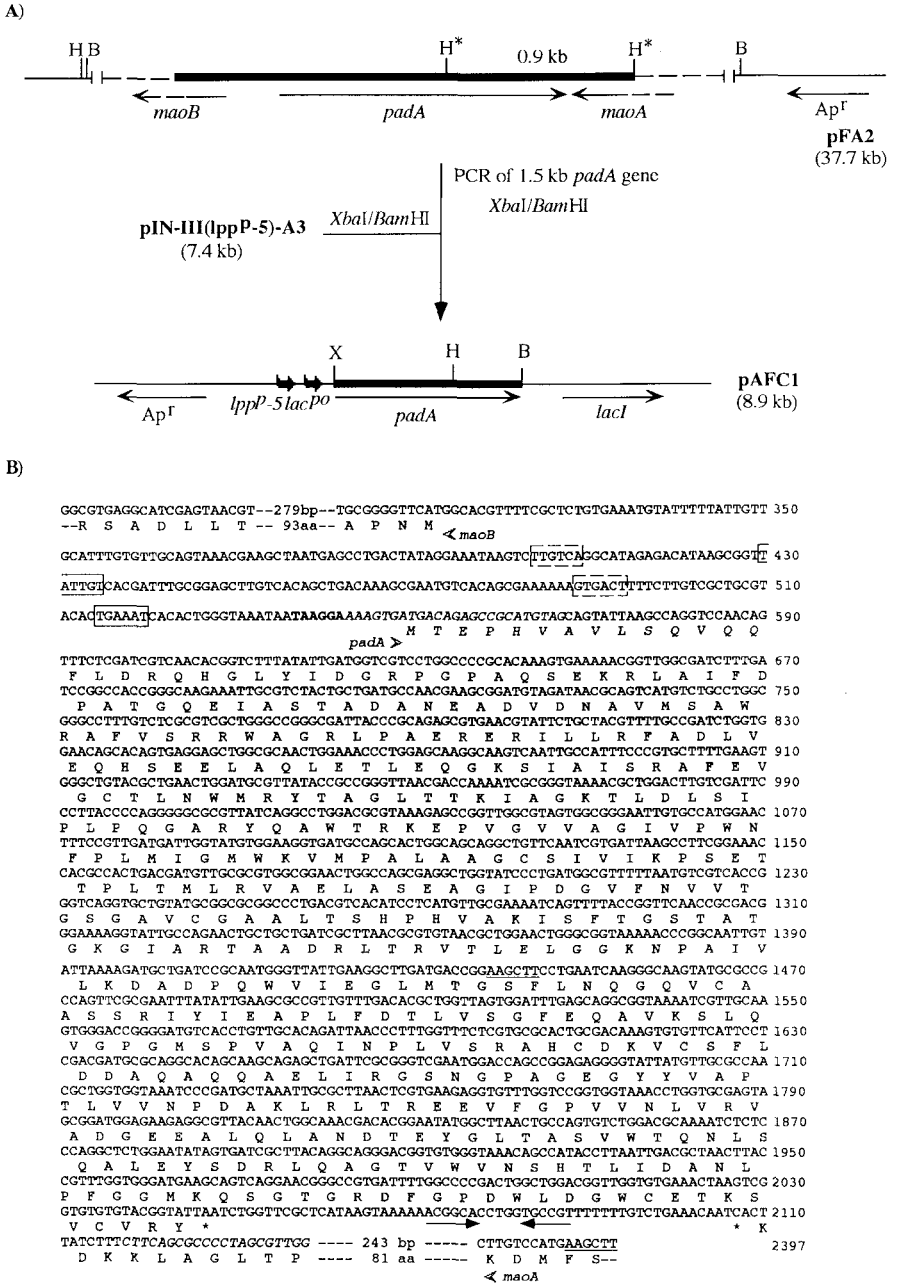

Fig. 2. A: Schematic representation of the cloning and overexpression of the *padA* gene. Thick lines indicate the 2397-bp chromosomal fragment whose nucleotide sequence has been determined. Thin and discontinuous lines represent the remaining 30.9-kb chromosomal insert (the drawing is not to scale). Thin and continuous lines mean vector derived sequences. Thin arrows indicate the direction of transcription of the genes. Thick arrows represent the modified lipoprotein promoter (lpp^{p}-*5*) and the lactose promoter operator (lac^{po}). Relevant restriction sites are shown: B, *Bam*HI; H, *Hin*dIII; X, *Xba*I. Asterisks mean that additional *Hin*dIII restriction sites are present in the 30.9-kb chromosomal insert (discontinuous line). Ap^{r} indicates the gene conferring ampicillin resistance. B: Nucleotide and derived amino acid sequences of the 2397-bp chromosomal insert. The sequence data appear in the GenBank/EMBL Data Bank with accession number X97453. Nucleotides 1–462 are complementary to nucleotides 1–462 of sequence D67041 [7]; nucleotides 2020–2397 are complementary to nucleotides 2907–3281 of sequence D23670, although we have found 3 substitutions in the *maoA* gene as well as 4 nucleotide insertions and the deletion of 1 nucleotide in the *maoA-padA* intergenic region (data not shown). Amino acids are represented in one-letter code. Only a partial sequence of the *maoB* and *maoA* genes is shown. The *Hin*dIII restriction sites are underlined. The inverted repeat of a potential ρ-independent transcription terminator is marked with convergent arrows underneath the sequence. Arrowheads show the direction of gene expression. Asterisks indicate the stops condons. Boldface nucleotides represent potential Shine-Dalgarno sequences. Putative −10 and −35 promoter sequences are boxed with continuous and discontinuous lines, respectively. The nucleotide sequences present in the primers used for PCR amplification of *padA* (A) are indicated in italics.

these data indicate that alkaline pH values are optimal for in vitro PadA activity, as is also the case for most aldehyde dehydrogenases [21,22,25].

The substrate specificity of PadA was also examined using several aliphatic and aromatic aldehydes. As expected from the suggested physiological role of this enzyme, the maximum activity was observed with PAL. We did not observe PadA activity when using acetaldehyde and propionaldehyde as sub-

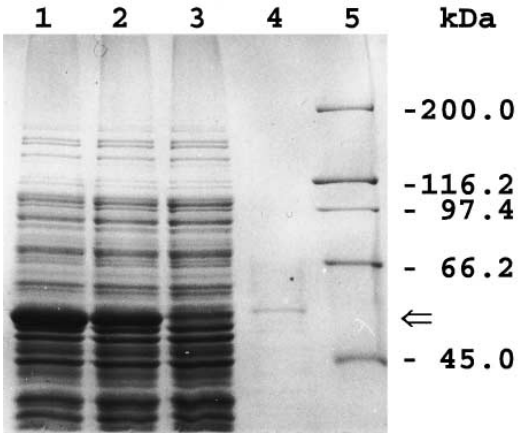

Fig. 3. 8% SDS-PAGE analysis of PadA. Lane 1, IPTG-induced DH5α cells containing plasmid pAFC1 (whole cell lysate); lane 2, non-induced DH5α cells containing plasmid pAFC1 (whole cell lysate); lane 3, IPTG-induced DH5α cells containing plasmid pIN-III(lpp^{p}-5)-A3 (whole cell lysate); lane 4, PadA protein after FPLC gel filtration chromatography; lane 5, molecular mass markers (Bio-Rad) (their molecular mass is indicated on the right in kDa). The arrow shows the position of the PadA protein.

strates. With regard to aromatic aldehydes, while benzaldehyde and *trans*-cinnamaldehyde were not substrates, phenylpropionaldehyde was oxidized although with a catalytic efficiency about 15-fold lower than that for PAL. Furthermore, by measuring in crude extracts of *E. coli* the formation of NADH through the combined action of the 2-phenylethylamine oxidase and phenylacetaldehyde dehydrogenase, Parrott et al. [2] have shown that the aldehydes derived from tyramine and dopamine, i.e. 4-OH-PAL and 3,4-OH-PAL, respectively, could also be substrates of this dehydrogenase. A high specificity for PAL-like aromatic aldehydes has also been observed for the phenylacetaldehyde dehydrogenase of *A. eurydice* [1], and is consistent with the physiological role and the reported specific induction of PadA by 2-phenylethylamine [2].

Finally, the PadA enzyme was also able to catalyze the hydrolysis of *p*-nitrophenyl acetate, but this esterase activity (5.6 nmol/min per mg protein) was less than 0.3% of the dehydrogenase activity. Although an esterase activity of 1–15% of the dehydrogenase activity is a property of many aldehyde dehydrogenases, its physiological significance is still unknown [25].

In summary, the results presented in this work provide genetic, structural, and biochemical data on the *E. coli* PadA enzyme, a new member of the superfamily of aldehyde dehydrogenases, and constitute the first report on the nucleotide sequence of a gene encoding a phenylacetaldehyde dehydrogenase. Since PAL is an intermediate in the catabolism of toxic aromatic compounds [4,5], the *padA* gene provides a new catabolic tool to increase by in vitro pathway design the ability of microorganisms to degrade aromatic pollutants.

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