ELSEVIER

FEMS Microbiology Letters 202 (2001) 145–148

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Characterization of a new feedback-resistant 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase AroF of *Escherichia coli*

Ralf Jossek [a], Johannes Bongaerts [a,b], Georg A. Sprenger [a,*]

[a] *Institut für Biotechnologie 1, Forschungszentrum Jülich GmbH, D-52425 Jülich, Germany*
[b] *DSM Biotech GmbH, Karl-Heinz-Beckurts-Str. 13, D-52428 Jülich, Germany*

Received 16 March 2001; accepted 26 June 2001

First published online 17 July 2001

Abstract

Tyrosine feedback-inhibits the 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase isoenzyme AroF of *Escherichia coli*. Here we show that an Asn-8 to Lys-8 substitution in AroF leads to a tyrosine-insensitive DAHP synthase. This mutant enzyme exhibited similar activities ($v = 30$–40 U mg^{-1}) and substrate affinities ($K_m^{\text{erythrose-4-phosphate}} = 0.5$ mM, positive cooperativity with respect to phospho(enol)pyruvate) as the wild-type AroF, but showed decreased thermostability. An engineered AroF enzyme lacking the seven N-terminal residues also was tyrosine-resistant. These results strongly suggest that the N-terminus of AroF is involved in the molecular interactions occurring in the feedback-inhibition mechanism.

Keywords: 3-Deoxy-D-arabino-heptulosonate synthase; Feedback-inhibition; AroF; Tyrosine resistance; *Escherichia coli*

1. Introduction

The enzyme 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHP synthase, EC 4.1.2.15) catalyzes the condensation of phospho(enol)pyruvate (PEP) and erythrose-4-phosphate (E4P) to DAHP and inorganic phosphate. This reaction is the first committed step in the biosynthesis of aromatic compounds in microorganisms and plants. DAHP synthase serves as the primary site for feedback regulation of carbon flow into the shikimate pathway. *Escherichia coli* has three DAHP synthase isoenzymes, each of which is feedback-regulated by one of the aromatic amino acids, tyrosine, phenylalanine, and tryptophan. The enzymes are encoded by the structural genes *aroF* (tyrosine-sensitive DAHP synthase), *aroG* (phenylalanine-sensitive), and *aroH* (tryptophan-sensitive) [1]. The polypeptide chains of the DAHP synthase isoenzymes are similar in size (356, 350 and 348 amino acids, respectively) and share a high degree of sequence similarity. The identical residues are mainly located in central regions of the sequences (e.g. amino acid residues 50–192), whereas significantly less similarity is found in the C- (46 identical residues out of the last 157) and N-terminus (six identical residues out of the first 41) [2,3].

Several mutational changes result in feedback-resistant types of DAHP synthases [2,4–6]. All mutations are localized in three interior regions (residues 18, 146–154, and 178–180). From the crystal structure of AroG it is supposed that these regions are involved in the formation of a putative inhibitor-binding site [7]. Here we demonstrate the involvement of the N-terminus of the polypeptide chain of AroF in the feedback-inhibition mechanism of the DAHP synthase isoenzyme.

2. Materials and methods

2.1. Cloning and expression of aroF alleles

The *aroF* wild-type gene was amplified from *E. coli* K-12 strain W3110 [8], using primers aroF-up (5′-CTA TCG AAT TCG AGC ATA AAC AGG ATC GCC-3′) and aroF-down (5′-CAC TTC AGC AAC CAG TTC CCG

* Corresponding author. Tel.: +49 (2461) 61 6205, Fax: +49 (2461) 61 2710.
E-mail address: g.sprenger@fz-juelich.de (G.A. Sprenger).

Abbreviations: BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane; DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; E4P, erythrose-4-phosphate; IPTG, isopropyl-β-D-thiogalactopyranoside; PEP, phospho(enol)pyruvate

0378-1097/01/$20.00
PII: S0378-1097(01)00311-1

GGG CTT CGC-3′) generating a 1213-bp fragment with engineered *Eco*RI and *Sma*I restriction sites. The PCR product was gel-purified and cloned into *Eco*RI/*Sma*I-digested pBlueII KS(−) (Stratagene, La Jolla, CA, USA) to give pBII-*aroF*^{wt}. From this a 1191-bp *Eco*RI/*Bam*HI fragment containing *aroF*^{wt} was cloned in pJF119EH [9] to give pJF-*aroF*^{wt}. The gene *aroF*^{fbr} was obtained from DSM Biotech (Jülich, Germany) and was cloned into the *Eco*RI and *Bam*HI sites of plasmid pJF119EH to give pJF-*aroF*^{fbr}. Taking advantage of the *Pst*I site within the *aroF* gene, plasmid pJF-*aroF′* was obtained by replacing the 812 bp *Pst*I fragment of pJF-*aroF*^{wt} (comprising the second half of *aroF*) with the respective *Pst*I fragment of pJF-*aroF*^{fbr}. Plasmids pBII-*aroF*^{wt} and pJF-*aroF*^{fbr} were digested with *Pst*I/*Sca*I, and the 1.9-kb fragment of PbII-*aroF*^{wt} and the 4.8-kb fragment of pJF-*aroF*^{fbr} were ligated to result in pJF-*aroF*(N8K). The gene *′aroF* was amplified from *E. coli* W3110, using primers *′aroF*-up (5′-GAC GCG CTG ACC ATG GTA CAT ATT ACC GAC-3′) and *′aroF*-down (5′-GCG GTC AAT TCA GGA TCC ATA ATA AAC CTC-3′) generating a 1092-bp fragment with engineered *Nco*I and *Bam*HI restriction sites. The gel-purified PCR product was cloned in *Nco*I/*Bam*HI-digested pET16b (Novagen, Madison, WI, USA) to give pET16b-*′aroF*. From this, a 1393-bp *Xba*I/*Hin*dIII fragment containing *′aroF* was cloned in pJF119EH to give pJF-*′aroF*. The cloned genes were verified by sequencing using a LI-COR automatic sequencing system (MWG AG Biotech, Ebersberg. Germany). The various types of AroF were overexpressed in *E. coli* strain AB3257 (*aroG*365, *aroH*367, *aroF*363 as relevant markers) (CGSC strain # 3257) [10].

2.2. Assay of DAHP synthase activity

Enzyme activity was assayed by a modification of the method decribed by Schoner and Herrmann [11]. DAHP synthase was incubated in 50 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane (BTP) pH 6.8 containing 1 mM $FeSO_4$ and 0.5 mM PEP at 30°C. The reaction was initiated by the addition of 1.5 mM E4P (final concentration). DAHP synthase activity was measured photometrically at 232 nm in a thermostatted Beckman DU640 spectrophotometer by monitoring the rate of disappearance of PEP ($\varepsilon = 2800$). One unit of enzyme activity is defined as the disappearance of 1 μmol PEP per min.

2.3. Purification of DAHP synthases

AroF^{wt} and AroF(N8K) were purified by a four-step chromatography procedure derived from methods previously described [11,12]. All purification steps were carried out at 4°C in 10 mM BTP pH 6.8 containing 200 μM PEP, unless specified otherwise. Cells were grown overnight at 37°C in Luria–Bertani medium [13] containing 100 mg l^{-1} ampicillin and 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), harvested, washed with 0.9% NaCl and disrupted by sonication. Cell debris was removed by centrifugation at 40 000 × *g* for 60 min at 4°C. The supernatant was applied to a Q-Sepharose HP FPLC column (Pharmacia, Freiburg, Germany) and DAHP synthase was eluted at approximately 210 mM KCl. Fractions with high DAHP synthase activity were combined and unrelated proteins were precipitated by 1.25 M ammonium sulfate. The supernatant was loaded on a phenylsepharose HP FPLC column (Pharmacia, Uppsala, Sweden) and DAHP synthase was eluted at approximately 0.5 M $(NH_4)_2SO_4$. Active fractions were pooled, concentrated (Centriprep YM30, Amicon), and applied to a Superdex200 FPLC column (Pharmacia). Protein was eluted in 10 mM potassium phosphate, pH 6.8, containing 200 μM PEP. Fractions with DAHP synthase activity were combined and directly applied to a ceramic hydroxyapatite column (Bio-Rad, Munich, Germany). Elution was with a linear gradient of 10–500 mM potassium phosphate. Fractions with high DAHP synthase activity were pooled, and stored at −20°C.

3. Results and discussion

3.1. N-terminal modifications result in tyrosine-insensitive types of AroF

For the fermentative production of aromatic amino acids, the use of microbial strains expressing feedback-resistant DAHP synthases is a well known strategy to increase the carbon flow into the shikimate pathway [14]. The gene *aroF*^{fbr} encodes a feedback-resistant type of the tyrosine-sensitive DAHP synthase of *E. coli* [15]. Our sequence analysis revealed that the primary structure of AroF^{fbr} displays two modifications compared to AroF^{wt} (Fig. 1). These modifications are located at the N- and C-termini of the polypeptide chain of the molecule, respectively. The C-terminal modification consists of an exchange of the last five amino acids, and residue Asn-8 is substituted by Lys-8. The latter is the result of a single bp change in the *aroF* gene at nucleotide 23.

In order to investigate which of these two modifications caused the tyrosine-insensitive phenotype of AroF, we sep-

	1										10			350						356
AroF^{wt}	m	q	k	d	q	l	n	**N**	v	h	...			q	l	**T**	**A**	**R**	**V**	**A**
AroF^{fbr}	m	q	k	d	q	l	n	**K**	v	h	...			q	l	**P**	**R**	**A**	**F**	**R**
AroF′	m	q	k	d	q	l	n	**N**	v	h	...			q	l	**P**	**R**	**A**	**F**	**R**
AroF(N8K)	m	q	k	d	q	l	n	**K**	v	h	...			q	l	**T**	**A**	**R**	**V**	**A**
'AroF	-	-	-	-	-	-	-	**M**	v	h	...			q	l	**T**	**A**	**R**	**V**	**A**

Fig. 1. Comparison of the N- and C-terminal positions of the deduced amino acid sequences of the *aroF* alleles. The numbers indicate the positions of the amino acids in the polypeptide chain. Differences are shown in boldface and capital letters.

arated both modifications by cloning to yield *aroF′* and *aroF*(N8K). The plasmid-borne genes encoding AroF^{wt}, AroF^{fbr}, AroF′ and AroF(N8K), respectively, were introduced into the mutant *E. coli* strain AB3257 which completely lacks DAHP synthase activity [10]. DAHP synthase activities in cell-free extracts were assayed in the presence of 0–100 μM L-tyrosine. As shown in Fig. 2, DAHP synthase activity of wild-type AroF was inhibited to 50% in the presence of 20 μM tyrosine, in line with a previous report [16]. At 100 μM tyrosine, AroF^{wt} retained less than 10% residual enzyme activity. AroF′, carrying the C-terminal modification of the polypeptide, was similarly inhibited as wild-type AroF. In contrast, both AroF^{fbr} and AroF(N8K) were not inhibited by addition of 100 μM tyrosine. These results unambiguously demonstrate that a single substitution (Asn-8 to Lys-8) is sufficient to generate a tyrosine-insensitive form of AroF. To study further the role of the N-terminus of the polypeptide chain in feedback-inhibition of AroF, we engineered a DAHP synthase which lacks the first seven N-terminal residues (′AroF, Fig. 1). ′AroF DAHP synthase activity in cell-free extracts exhibited no sensitivity toward tyrosine in a range of 0–100 μM L-tyrosine (Fig. 2).

So far, several mutational changes resulting in feedback resistance of the three *E. coli* DAHP synthase isoenzymes have been described [2,4–6]. Eight of nine residues which have been found to be involved in feedback-inhibition are localized in two distinct regions, at positions 146–154 and 178–180, respectively. In addition, a change (Pro-18 to Leu-18) near the N-terminus of the polypeptide chain of the *E. coli* DAHP synthase AroH resulted in a tryptophan-insensitive type. Based on the apparent similarities in sequence and kinetic characteristics of the DAHP synthase isoenzymes, Shumilin and coworkers [7] had combined the results and transferred these to the three-dimensional structure of AroG. It was found that all feedback-resistant mutations are clustered in three regions, and are involved in the formation of a proposed inhibitor-binding site. Contrary to that, the N-terminal part of the polypeptide chain is distinct from the putative inhibitor-binding site of the molecule. It is noteworthy that in the case of AroG the N-terminal region of one subunit is closely adjacent to the proposed inhibitor-binding site of another subunit [7]. We hypothesized that modifications in the N-terminal part of the polypeptide chain could result in the exclusion of an inhibitor from the binding site.

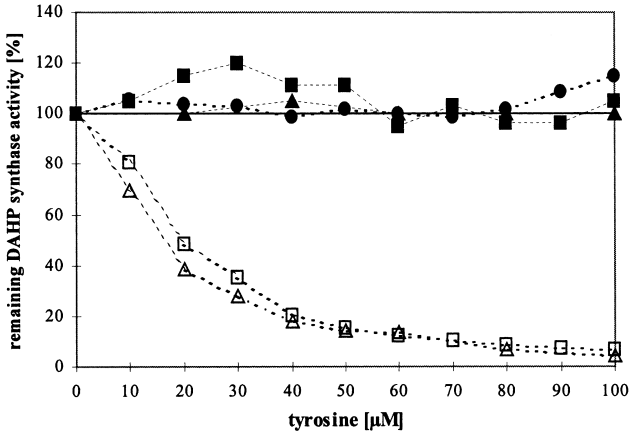

Fig. 2. Tryosine-inhibition patterns of DAHP synthases encoded by various *aroF* alleles. DAHP synthase activities of *E. coli* AB3257 harboring pJF-*aroF*^{wt} (□), pJF-*aroF*^{fbr} (■), pJF-*aroF′* (△), pJF-*aroF*(N8K)(▲), and pJF-′*aroF*(●), respectively, were measured in cell-free extracts in the presence of various amounts of added tyrosine. The data represent the average values of multiple measurements, the standard deviations are determined to be < 10%.

3.2. Biochemical charactization of AroF(N8K) and AroF^{wt}

To assay whether the replacement of Asn-8 by Lys-8 in AroF caused changes in the global protein structure or in interactions occurring in feedback-inhibition in particular, purified AroF(N8K) was characterized further. Since enzyme activity and substrate affinity of DAHP synthases vary greatly with the assay conditions (e.g. influence of PEP, trace metals and buffer) [12,16,17] AroF(N8K) was purified and characterized in parallel to AroF^{wt}. As aforementioned, in the three-dimensional structure of AroG the N-terminal part of one polypeptide chain is in proximity to the proposed inhibitor-binding site of a second subunit, but a direct involvement of the N-terminus in inhibitor binding has not been discussed [7]. It is considered that the N-terminal strand of one monomer interacts with two strands of another monomer to generate a tight dimer. Assuming a structural analogy of AroG and AroF it is conceivable that a mutational change in the N-terminus of the polypeptide chain of AroF causes a monomerization of the DAHP synthase resulting in feedback resistance. To analyze this the molecular masses of purified AroF(N8K) and AroF^{wt} were estimated by size exclusion chromatography on a Superdex200 FPLC column. By extrapolation from a standard curve, the molecular masses of both DAHP synthases were estimated to 71 000 and 75 000 (± 8000), respectively and were thus similar to native AroF^{wt} described by Schoner and Herrmann [11]. This is in favor of a homodimeric structure for both enzymes and demonstrates that the quaternary structure of AroF is not affected by the N8K mutation.

Changes in enzyme activity and substrate affinity indicate structural alterations in the active site and substrate-binding sites of the molecule, respectively. Therefore kinetic properties of purified AroF(N8K) in comparison to the wild-type AroF were determined. AroF(N8K) and AroF^{wt} exhibited similar specific DAHP synthase activities (30–40 U mg^{-1}) in the same range as reported before for tyrosine-sensitive DAHP synthase of *E. coli* (22–56 U mg^{-1}) [11,12,17]. The affinities of AroF(N8K) and AroF^{wt} for E4P and PEP were determined next. K_m values of 0.49 (± 0.07) mM and 0.48 (± 0.03) mM were obtained for AroF(N8K) and AroF^{wt}, respectively, using 0.5 mM PEP and 0.1–1.1 mM E4P. The velocity curves for Ar-

oF(N8K) and AroFwt in presence of 1.5 mM E4P and 0.1–0.5 mM PEP were sigmoidal indicating that both types of AroF do not obey simple Michaelis–Menten kinetics but display positive cooperativity concerning PEP, as described before for the kinetic properties of the phenylalanine- and tryptophan-sensitive DAHP synthase isoenzymes of *E. coli* [17,18]. For unknown reasons, our substrate K_m's for AroF^{wt} are significantly higher than those reported by others using similar preparations (0.1 mM [11]). In direct comparison, however, AroF(N8K) and AroF^{wt} displayed similar kinetic properties. This underlines that the N8K modification of AroF neither affects the active site of the molecule nor the mechanism of the enzyme reaction. Although similar in terms of molecular mass and kinetic characteristics, the mutant type of AroF is somewhat less stable at 37°C (a 20-min incubation in the presence of PEP led to a 50% decrease of DAHP synthase activity, whereas a similar loss of enzyme activity was observed only after 80 min at 37°C for AroF^{wt}) but it is unclear what the basis of the inactivation is. We demonstrate that modifications in the N-terminus of the polypeptide chain of AroF (either the N8K substitution or removal of seven amino acids in 'AroF) resulted in tyrosine-insensitive forms of the DAHP synthase. Thus, the N-terminus of AroF is involved in the molecular interactions occurring in the feedback-inhibition mechanism, a fact that had not been considered previously. Our results are in favor of the idea that the inhibitor specificity of the DAHP synthase isoenzymes of *E. coli* is rather provided by the C- or N-terminal domains than by the highly identical interior regions of the DAHP synthases [3]. Moreover, the concomitant resistance to feedback-inhibition and the decreased thermostability in the N8K mutein are apparently provoked by a single mutation. The structural changes in both mutant proteins with N-terminal modifications leading to feedback resistance, however, need to be elucidated in more depth.

Acknowledgements

We thank M. Berlyn (CGSC) for strain AB3257, DSM Biotech Jülich for the cloned *aroF*^{fbr} and *aroF'* alleles, and R. Halbach and U. Degner for expert technical assistance. We gratefully acknowledge Prof. H. Sahm for his continuous support and the BMBF for financial support through the BioRegio program.

References

[1] Pittard, A.J. (1996) Biosynthesis of aromatic amino acids. In: *E. coli* and *S. typhimurium*: Cellular and Molecular Biology (Neidhardt, F.C. et al., Eds.), Vol. 1, pp. 458–484. Am. Soc. Microbiol., Washington, DC.

[2] Ray, J.M., Yanofsky, C. and Bauerle, R. (1988) Mutational analysis of the catalytic and feedback sites of the tryptophan-sensitive 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase of *Escherichia coli*. J. Bacteriol. 170, 5500–5506.

[3] Shultz, J., Hermodson, M.A., Garner, C. and Herrmann, K.M. (1984) The nucleotide sequence of the *aroF* gene of *Escherichia coli* and the amino acid sequence of the encoded protein, the tyrosine-sensitive 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase. J. Biol. Chem. 259, 9655–9661.

[4] Ger, Y.-M., Chen, S.-L., Chiang, H.-J. and Shiuan, D. (1994) A single Ser-180 mutation desensitizes feedback inhibition of phenylalanine-sensitive 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthetase in *Escherichia coli*. J. Biochem. 116, 986–990.

[5] Kikuchi, Y., Tsujimoto, K. and Kurahashi, O. (1997) Mutational analysis of the feedback sites of phenylalanine-sensitive 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase of *Escherichia coli*. Appl. Environ. Microbiol. 63, 761–762.

[6] Weaver, L.M. and Herrmann, K.M. (1990) Cloning of an *aroF* allele encoding a tyrosine insensitive 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase. J. Bacteriol. 172, 6581–6584.

[7] Shumilin, I.A., Kretsinger, R.H. and Bauerle, R.H. (1999) Crystal structure of phenylalanine regulated 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase from *Escherichia coli*. Structure 7, 865–875.

[8] Bachmann, B.J. (1972) Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. 36, 525–557.

[9] Fürste, J.P., Pansegrau, W., Frank, R., Blocker, H., Scholz, P., Bagdasarian, M. and Lanka, E. (1986) Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tac*P expression vector. Gene 48, 119–131.

[10] Wallace, B.J. and Pittard, J. (1969) Regulation of 3-deoxy-D-*arabino*-heptulosonic 7-phosphate acid synthase activity in relation to the synthesis of the aromatic vitamins in *Escherichia coli* K-12. J. Bacteriol. 99, 707–712.

[11] Schoner, R. and Herrmann, K.M. (1976) 3-Deoxy-D-*arabino*-heptulosonate 7-phosphate synthase. Purification, properties, and kinetics of the tyrosine-sensitive isoenzyme from *Escherichia coli*. J. Biol. Chem. 251, 5440–5447.

[12] Ramilo, C.A. and Evans, J.N.S. (1997) Overexpression, purification, and characterization of tyrosine-sensitive 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate synthase from *Escherichia coli*. Protein Expr. Purif. 9, 253–261.

[13] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) In: Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[14] Frost, J.W. and Draths, K.M. (1995) Biocatalytic syntheses of aromatics from D-glucose: Renewable microbial sources of aromatic compounds. Annu. Rev. Microbiol. 49, 557–579.

[15] Krämer, M. (2000) PhD Thesis, University of Düsseldorf, Düsseldorf.

[16] Dusha, I. and Dénes, G. (1976) Purification and properties of tyrosine-sensitive 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase of *Escherichia coli* K12. Biochim. Biophys. Acta 438, 563–573.

[17] Stephens, C.M. and Bauerle, R. (1991) Analysis of the metal requirement of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase from *Escherichia coli*. J. Biol. Chem. 266, 20810–20817.

[18] Akowski, J.P. and Bauerle, R. (1997) Steady-state kinetics and inhibitor binding of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (tryptophan sensitive) from *Escherichia coli*. Biochemistry 36, 15817–15822.