An operon encoding aspartokinase and purine phosphoribosyltransferase in *Thermus flavus*

Makoto Nishiyama,¹ Mutsuko Kukimoto,² Teruhiko Beppu^{1†} and Sueharu Horinouchi²

Author for correspondence: Makoto Nishiyama. Tel: +81 3 3812 2111 ext. 3072. Fax: +81 3 5802 3326.

Biotechnology Research Center¹ and Department of Biotechnology², The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113, Japan

The nucleotide sequence of a 1.1 kb *Xho*I–*Hind*III fragment downstream of the malate dehydrogenase (*mdh*) gene of *Thermus flavus* revealed the presence of an ORF and an incomplete ORF lacking its NH₂-terminal portion, in the opposite orientation to that of the *mdh* gene. These two genes overlapped with each other, sharing two base pairs, suggesting that these genes are co-transcribed in a single mRNA. One ORF (termed *gpt*) encoded a protein of 154 amino acids showing significant amino acid sequence similarity to purine phosphoribosyltransferases, such as xanthine-guanine phosphoribosyltransferase of *Escherichia coli* and human hypoxanthine phosphoribosyltransferase. Cloning and sequencing of the upstream region of the *gpt* gene, together with sequence comparison of the gene product encoded by the region upstream of *gpt*, suggested that the upstream ORF encoded two in-frame overlapping aspartokinase genes, *askA*, encoding the α -subunit of 405 amino acids, and *askB*, encoding the β -subunit of 161 amino acids, which was part of the 3' portion of *askA*. Consistent with the sequence data, the *askAB* and the *gpt* genes conferred the heat-stable enzyme activities of aspartokinase and phosphoribosyltransferase, respectively, on *E. coli*. Preliminary characterization of these enzymes produced in *E. coli* is described.

Keywords: *Thermus flavus*, aspartokinase, end-product inhibition, purine phosphoribosyltransferase, thermostable enzyme

INTRODUCTION

We previously reported that the *mdh* gene encoding malate dehydrogenase in *Thermus flavus* AT-62 is transcribed as an operon containing the *scsAB* genes encoding the α - and β -subunits of succinyl-CoA synthetase, in addition to the *mdh* gene (Iijima *et al.*, 1986; Nishiyama *et al.*, 1986, 1991). In *Escherichia coli*, however, the *mdh* gene is transcribed in a monocistronic manner (Darlison *et al.*, 1984) and is located at a genetic map position different from that of the major gene cluster for the enzymes involved in the tricarboxylic acid cycle (Darlison & Guest, 1984; Spencer *et al.*, 1984; Wood *et al.*, 1984; Buck *et al.*, 1985; Heard *et al.*, 1975; Southerland & McAlister-

Henn, 1985), which includes the *sucCD* genes encoding two subunits of succinyl-CoA synthetase. To clarify further the gene arrangement of the *mdh* locus of *T. flavus*, we determined the nucleotide sequence of the region downstream of *mdh*. This analysis revealed the presence of genes encoding aspartokinase (*askAB*) and purine phosphoribosyltransferase (*gpt*), which are responsible for the biosynthesis of branched-chain amino acids and salvage synthesis of purine nucleotides, respectively. This paper describes the cloning and sequence analysis of the aspartokinase and the phosphoribosyltransferase (PRT) genes downstream of the *mdh* gene of *T. flavus*, along with preliminary characterization of the gene products.

METHODS

Enzymes and chemicals. Restriction endonucleases, T4 DNA ligase, and DNA polymerase I (Klenow fragment) were purchased from Takara Shuzo. [α -³²P]dCTP was from Amersham Japan. A kit for nucleotide sequencing by the M13-dideoxy nucleotide method (Sanger *et al.*, 1977) was obtained from USB. Oligonucleotides used were synthesized with a Cyclone Plus DNA synthesizer (Millipore).

† **Present address:** Department of Applied Biological Science, College of Agriculture and Veterinary Medicine, Nihon University, Kameino, Fujisawa, Kanagawa 252, Japan.

The GSDB/DBJ/EMBL/NCBI accession number for the nucleotide sequence reported in this paper is D37928.

Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase; PRT, phosphoribosyltransferase; RBS, ribosome-binding site; XGPRT, xanthine-guanine phosphoribosyltransferase.

Bacterial strains and plasmids. *E. coli* JM105 [$\Delta(lac-pro)$ *thi strA endA sbcB15 hsdR4 F' traD36 proAB lac^r lacZAM15*] was used as a host for the expression of the *gpt* and *ask* genes and also for M13 phage propagation for determination of the nucleotide sequence.

Plasmid pTA101 (Iijima *et al.*, 1986), which contains a 3.2 kb *HindIII* fragment carrying the *mdh* gene of *T. flavus* AT-62 was used as a DNA source for sequencing and chromosome walking.

Plasmid pPRT101 for the expression of the *gpt* gene was constructed as follows. pUC19 digested with *Bam*HI was treated with Klenow fragment and then digested with *Sac*I. The resulting DNA fragment was ligated with the 1617 bp *Stu*I–*Sac*I fragment which covers the whole *gpt* coding region to yield pPRT101. This plasmid allowed the expression of *gpt* under the control of the *lac* promoter of *E. coli*, where an AGAGG sequence in the multiple cloning cartridge, located 7 bp upstream of the putative translation initiation codon ATG for the *gpt* gene, could serve as the ribosome-binding site (RBS) for translation.

For expression of the *askAB* genes in *E. coli*, plasmid pAKT101 was constructed as follows. PCRs were done using pDMD101 constructed in this study as a template and two synthetic oligonucleotides as primers, 5'-CGAATTCCTAAAAGGAG-GGAAGCATGGCCCT-3' and 5'-CAGCATCTGGTCGTA-GCCGATGAC-3'. The former was designed to contain an *Eco*RI site just upstream of the putative RBS. This primer was also designed to maximize translational efficiency by replacing GTG of the *askA* gene with ATG as the initiation codon. The latter was derived from the AskA-encoding sequence (nt 787–810, see Fig. 1). The PCR conditions were 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, with a total of 20 cycles. After digestion of the amplified DNA with *Eco*RI and *Hind*III, the resulting restriction fragment of about 600 bp was ligated with the 860 bp *Hind*III–*Pst*I fragment from pDMD101 and pUC18 digested with *Eco*RI plus *Pst*I to yield pAKT101.

Another expression plasmid, pAKT102, was constructed as follows. Two synthetic oligonucleotides, 5'-GGCTCTAGAT-TAGGCCTTGTCAGCTCAAAAGC-3', which was designed to introduce an *Xba*I site just downstream of the stop codon TAA for the aspartokinase genes, and 5'-CACCTGA-TCCCCGAGGCGAGGAAG-3' (nt 757–780) were used as the primers for PCR with pDMD101 as the template, and the amplified DNA fragment was digested with both *Hind*III and *Xba*I. Additional PCRs using the oligonucleotides 5'-CCTCT-AGAAAGGAGGTGGACGATGGAGATGGACAAGGCG-GTGACG-3' and 5'-GGGCATGCTCTATCAGGCCTTGT-CCAGCTC-3', were carried out to introduce *Xba*I and *Sph*I restriction sites to the 5' and 3' termini of the *askB* gene, respectively, and the resulting amplified DNA was digested with both *Xba*I and *Sph*I. The two restriction fragments thus prepared by PCR were ligated with the ~600 bp *Eco*RI–*Hind*III fragment from pAKT101 containing the NH₂-terminal portion of the α -subunit of aspartokinase, and with pUC18 digested with both *Eco*RI and *Sph*I, and were introduced into *E. coli* JM105. The resulting plasmid, named pAKT102, contained the *askAB* genes and an additional copy of the *askB* gene. The nucleotide sequence of the junction region between the *askAB* and the additional *askB* genes was 5'-gccTAAtctagaaggagggtggacgATGgagatg-3' (capitalized TAA and ATG represent the termination codon of *askAB* and the initiation codon of *askB*).

DNA manipulation. All the enzymes used for DNA manipulation were purchased from Takara Shuzo. Total chromosomal DNA of *T. flavus* was prepared by the method of Saito & Miura (1963). For cloning the whole coding region of the *askA* gene, the 330 bp *Hind*III–*Xba*I fragment containing a central portion

of the aspartokinase gene was prepared by agarose gel electrophoresis and used as a probe for Southern hybridization (Southern, 1975). Colony hybridization was carried out by the method of Grunstein & Hogness (1975). The nucleotide sequence was determined by the dideoxy chain-termination method using M13 phages (Sanger *et al.*, 1977; Messing, 1983). All restriction sites used for cloning on M13 RFI DNA were verified by determination as part of an overlapping sequence.

Computer analysis of amino acid sequences. Amino acid sequence similarity was analysed using the protein databases of National Biomedical Research Foundation and EMBL with the GENETYX-CD software (Software Development).

Expression of *gpt*. *E. coli* cells harbouring pPRT101 were precultured in 10 ml 2 × YT medium (Yanisch-Perron *et al.*, 1985) supplemented with 50 µg ampicillin ml⁻¹ at 37 °C. Pre-culture (1 ml) was transferred to 100 ml of the same medium and cultured for 2 h. IPTG was then added to give a concentration of 1 mM and cultivation was continued for an additional 12 h. The cells were harvested by centrifugation, suspended in 4 ml 100 mM potassium phosphate buffer (pH 7.0), and disrupted by sonication (Branson Sonifier, cell disrupter model 250). After centrifugation of the sonicate at 10000 *g* for 30 min, the supernatant was heated at 70 °C for 30 min, which completely inactivated the purine PRT activity present in the *E. coli* host cells. The crude enzyme solution was obtained after centrifugation of the heated extract at 10000 *g* for 30 min.

Expression of *askAB*. *E. coli* cells carrying pAKT101 or pAKT102 were precultured at 37 °C in 10 ml 2 × YT medium containing 50 µg ampicillin ml⁻¹. Pre-culture (100 µl) was transferred to 10 ml of the same medium supplemented with 1 mM IPTG and cultivation was continued for another 12 h. The cells were harvested by centrifugation, suspended in 500 µl 100 mM Tris/HCl buffer (pH 7.5), and disrupted by sonication. The sonicates were then centrifuged at 16000 *g* for 10 min and heated at 70 °C for 30 min. After centrifugation of the heat-treated solution at 16000 *g*, the supernatant was used as the crude enzyme solution.

Enzyme assay. PRT activities were assayed at 37 °C. The reaction mixture contained 10 mM MgCl₂, 20 mM NaF, 5 mM 5-phosphoribosyl 1-pyrophosphate, 1.25 mM of each purine base (xanthine, hypoxanthine or adenine), and an appropriate amount of heat-treated cell extract in 100 mM Tris/HCl (pH 7.0). For determination of activity with guanine, the concentration of guanine in the reaction mixture was set at 0.5 mM due to the lower solubility of this compound in water. After an appropriate period of incubation, the reaction was stopped by 20-fold dilution with distilled water. The conversion of the purine base to the corresponding nucleotide monophosphate was analysed by HPLC (Shimadzu) equipped with a Senshu ODS VP-1151 column (4.6 × 150 mm) using 100 mM KH₂PO₄ (pH 5.0) as eluent at a flow rate of 1.0 ml min⁻¹ at 50 °C. The amounts of the substrates and products were estimated by measuring the absorbance at 260 nm. One unit (U) of enzyme activity was defined as the amount of enzyme which catalysed the formation of 1 µmol nucleotide min⁻¹ from the corresponding purine. Proteins were determined by the method of Lowry or of Bradford (1976).

Aspartokinase activity was determined by the method of Cohen (1985) with some modifications. The reaction mixture of 780 µl, which contained an appropriate amount of enzyme preparation, 0.9 M KCl, 6.4 mM ATP, 54 mM MgSO₄ · 7H₂O and 13 mM potassium L-aspartate in 0.45 M Tris/HCl buffer (pH 7.4), was incubated at 65 °C for 5 min. The reaction was then stopped by rapid cooling in ice-water. Phosphoenolpyruvate (100 µl) and NADH (100 µl) were added to give a concentration of 0.1 mM

of each and the reaction mixture was preincubated at 37 °C for 3 min. Lactate dehydrogenase (5 µl, rabbit muscle type II, 50 U, Sigma) and pyruvate kinase (5 µl, rabbit skeletal muscle type II, 10 U, Sigma) were then added to the reaction mixture, and the decrease in A_{340} due to the decrease in NADH was monitored using a spectrophotometer (Milton Roy Spectronic 3000 array or Shimadzu 160A). One unit was defined as the amount of enzyme which catalysed the oxidation of 1 µmol NADH min⁻¹ under the conditions described above. To determine the thermostability of aspartokinase, the remaining activity after heat treatment of the enzyme preparation at various temperatures for 30 min was measured. Inhibition of the aspartokinase activity by lysine and threonine was examined by adding the amino acids to the reaction mixture at a concentration of 1 mM.

RESULTS

Nucleotide sequence of the region downstream of *mdh* from *T. flavus*

We had already determined the nucleotide sequence of the 2.1 kb *Hind*III-*Xho*I segment of the 3.2 kb *Hind*III fragment cloned in pTA101, containing the *mdh* gene from *T. flavus* (Nishiyama *et al.*, 1986). In the present study, the 1.1 kb *Xho*I-*Hind*III fragment located downstream of *mdh* was sequenced and two ORFs in the opposite orientation to that of the *mdh* operon were found (Fig. 1). An ORF of 154 amino acids (17 103 Da) started from the initiation codon ATG (nt 1425–1427), and was preceded by a putative RBS, GGACAAG, with a 5 bp space, and terminated at TAA (nt 1887–1889). By computer-aided analysis, this ORF was found to encode a protein with significant sequence similarity to that of the xanthine-guanine PRT (XGPRT) of *E. coli* (Pratt & Subramani, 1983), and the human hypoxanthine PRT (HPRT) (Jolly *et al.*, 1983) (Fig. 2a). We concluded that this ORF encoded a purine PRT of *T. flavus*, because, as will be described below, this ORF specified heat-stable purine PRT activities in *E. coli*. We therefore designated the gene encoding this ORF as *gpt*. Another ORF (nt 778–1422) was found within the sequenced region but was obviously incomplete because of the absence of a start codon and RBS. However, the truncated ORF was found to code for a COOH-terminal portion of an enzyme whose amino acid sequence showed significant similarity to those of aspartokinases from *E. coli* (Katinka *et al.*, 1980; Zakin *et al.*, 1983; Cassan *et al.*, 1986), *Bacillus subtilis* (Chen *et al.*, 1987), and *Corynebacterium glutamicum* (Kalinowski *et al.*, 1991). Because of the high similarity of the *Thermus* aspartokinase to those of *B. subtilis* and *C. glutamicum*, we expected that it might also be encoded by in-phase overlapping genes, as is the case for the aspartokinases from these Gram-positive bacteria. We therefore tentatively named the gene(s) *askA* and *askB* for the large (α) and the small (β) subunits, respectively.

Structure of *askAB* from *T. flavus*

Since the 3.2 kb *Hind*III fragment which had already been cloned contained the whole *askB* coding region, but appeared to lack the complete *askA* gene, chromosome walking was used to obtain a DNA fragment containing

the 5' terminal portion of the *askA* gene. When the *T. flavus* chromosomal DNA digested with several restriction endonucleases was subjected to Southern hybridization with a *Hind*III-*Xho*I fragment (about 330 bp) containing a central part of the *askA* gene as the probe, a single *Pst*I fragment of approximately 1.7 kb gave positive hybridization (data not shown). The 1.7 kb *Pst*I fragment was recovered from agarose gels, inserted into the *Pst*I site of pUC19, and introduced into *E. coli* JM105. Three colonies among several hundred transformants gave positive hybridization with the probe, and the plasmids recovered from the three positive transformants possessed an identical 1.7 kb *Pst*I fragment containing the *askAB* genes from *T. flavus*. One of the these plasmids was designated pDMD101. A long ORF (*askA*) of 405 amino acids (43 318 Da) was identified by sequencing the cloned *Pst*I fragment; there is a potential RBS, AAGGAGG, 5 bp in front of the putative initiation codon GTG of the *askA* gene (Fig. 1).

When the deduced amino acid sequence of *Thermus* aspartokinase was compared with several other aspartokinases that were already sequenced, stronger similarity was found with aspartokinases from Gram-positive bacteria, such as *C. glutamicum* aspartokinase (46%) and *B. subtilis* aspartokinase II (45%), than with *E. coli* aspartokinase III (30%) (Fig. 2b). The aspartokinases of *B. subtilis* and *C. glutamicum* are tetrameric, and are composed of equal amounts of the α - and the β -subunits ($\alpha_2\beta_2$), encoded by in-phase overlapping genes. In both cases, the initiation codon for the β -subunit is located within the α -subunit-encoding region, and is preceded by a typical RBS. A similar situation for the β -subunit of *T. flavus* was observed: a methionine codon at the position corresponding to the putative initiation codon for the β -subunit was present and preceded by a potential RBS, AAGGAGG, with a 5 bp space. This suggested that the aspartokinase of *T. flavus* was composed of two subunits encoded by in-phase overlapping genes, as is the case for those of *B. subtilis* and *C. glutamicum*. These findings strongly suggested that the *askB* gene corresponded to the 3' portion of *askA* and that it coded for a protein of 161 amino acids (16 894 Da).

A possible promoter sequence (nt 138–166, 5'-TTGGGC-17 bp-TAACAT-3') is found 41 bp upstream of the initiation codon for *askA*. The termination codon for the *askAB* genes, TGA, overlapped with the putative initiation codon, ATG, of the *gpt* gene, sharing two bases, T and G. In addition, inverted repeat sequences capable of forming stable stem-loop structures, probably serving as ρ -independent transcriptional terminators, were found downstream of the *gpt* gene. These observations suggest that the *askAB* and *gpt* genes are co-transcribed in a single mRNA as an operon.

An additional ORF was found in the region upstream of the *askAB* genes in the opposite orientation to that of the *askAB* genes (Fig. 1). The amino acid sequence of this ORF, however, showed no sequence similarity to the protein sequences deposited in the EMBL or the NBRF databases.

Fig. 1. Nucleotide sequence of the genes encoding aspartokinase and purine PRT from *T. flavus*. Converging arrows represent inverted repeat sequences.

(a)

TFGPT	M-ERLHLSWEDLLRLVRRLAEAL-SGEFFDLVLGVARGGLIPTALLAQAAG	49
XGPRT	*S*YIIVT*DM*QQA*H*SR*HPS*QMKGIIA*S*V*G*RE**	51
HPRT	ATRSFGVSIIDDEPGYDLDFLFCIPNHYAEDLERVFIPIHG*IMDRTE*A*DVNKE-MG*HH-IVA*C*LK*YKFF*S*LSYIK	82
TFGPT	ARNILTAAV-----MFYEGERALPEPVLFQFPDVLFGKRVLVVDVDSGRFAFAVKARVRA-GGVVAVATLHFK	120
XGPRT	I*HVD*VCI-----SS*DDMDQREKL*AKRAEG*GEG-----I*I*LV*V*G*V*IREMYPK*-----HFV*IFA*	114
HPRT	*L*RVSDRSIPMTVDIFIRLKYCNDQSTGDIK*I-GGDDLST*TKKVV*IVE*II*T*K*MQTLISL*QYMPKMKV*S*LV*	164
TFGPT	P-GRNRVDPDRDPFYAKETAAMVVYPWALEAMPKG	154
XGPRT	*A*PL*D*Y---VVDIPQDT*IEQ*DMGVVFPFISGR	152
HPRT	R-TPRS*GYK**VGF*IPDKF*VGY*DYNEYFRDLNHVCVISGTGKAKYA	217

(b)

TFAK	MALVVG-----KYGOTSVDGLERIKHVAQRIAHYREKGRHLAVVVSANGHTDELIALAK-----	55
CGAK	*****LESAA*RN*E*VATKKA*HDVV*****D*****LE**A-----	55
BSAKII	*G*I*-----F*****SV*K*QNA*H*ALAEKQ**QVV*****S*****VS**	55
ECAKIII	*SEI*VS-----F*****A*PDAMNRS*DIVLSDA-HV-***L*SAGI*HL*V***E-----GLEPCERFEKLOAIRN	69
TFAK	-----RVMPRPFPFRLDLLTTGQVSVALLSMQIMANGIPAKGVQBGIGI	102
CGAK	-----A***V**A**H*N*LA*RI**H*VA*AIESL*AE*QS*GG*V*	102
BSAKII	-----AISDQ*SK**H*N*LA*****TIS*****A*QEK*YD*VSTGW*A*	102
ECAKIII	-IQFALLERLR--YP-VWIREIERLEENIT-VLAEEAALATS*-ALT*E*VSH**LM*TL*FVEI*REEDVQ*QW*DVNKV-M	146
TFAK	TTDGRYDARILEVNP*-RREAL--DQGFVAVI-AGFNGTPE-GEITTLGRGSDTTAVALAALGAKECIYTTEGVYT	180
CGAK	L*TE*H**Q*VD*V*G-----E*KICIV-***Q*VHK*TRDV*****L*****DV*****V*VD***	181
BSAKII	R*EALH*H**TDIDTS--VLADQ*-EK*IVIV-***Q*H*ED-C*****L*****KVDR*D*****VP*P*	180
ECAKIII	R*ND*P*R*E-PDIAALAEALAQ*LPRLNEGL*TO*I*SEKK*RT*****Y*ALL*E*H*SRVD*W*VP*I*	228
TFAK	TDPELIPKARKLSVIGYDQMLENAALGARVLSPRAVYIAKRYGVVLESRFSYH-PGTLVKE---VAMBDKAVTGVALDLD-	259
CGAK	A**RIV*H**Q*EKLSPFE**I*V*SKI*VL*S*E*RAFN*P*R*****D-----TAGSHEDIPVEAVL***T*HS-	262
BSAKII	**RYVKS***EG*S*E**L*H**G*****EF*LM*Q*P*E*****TETE-A**TE---ESS*QKLI*R*I*PEDQ-	260
ECAKIII	**RVVSA*KRIDE*AFARAA**TF*K**ATLLP*V*SDIPVF*G*KDPRAG**CC---XTENPPLFRALAKRMT	309
TFAK	-HAQILIGLIGIDOPGIAAKVFOALAEAGIADVMIIQGVGHPDPSRQMAFT-VKIDFAQA-LEALEPVLAEIGGEAIL-RPDI	339
CGAK	-E*KVTVL**S*K*E*****RG**DAE*NI*HVL*H*SSVEDGTTDIT**CPRSDGR*-H*I*KKLQVQGWVTV*YDDQV	343
BSAKII	-ITRVTVI*LTSLTLSTI*TT**KRN*N*I*-----TQAEKKTGIS*S**TED*QD*-VAV*ETKDALET*K*ETESKL	339
ECAKIII	LTLHS*H-MLHSR*FL*E*GI**RHN*S*L*TTSEVSVALTLDTG*STGDTLLTQSL*ME*-SK*CRVEV*EG*-----	386
TFAK	AKVSVIGVGLASTPEVPAKOHFOAVASTGANIEMIATSEVRISVIIIPAYAAALRAVH-QAFELDKA	405
CGAK	G**L*L*A*HK*H*G*TFE*E*LRDVNV**L*S**I**L*REDDLD*A*LI*-EQ*Q*GGEDAVVYAGTR	421
BSAKII	*****S*HV*N*G*A*E*AVL*QKNIL*LV*S**IKV*TVVSEMDNVK*VESL*-D***S*EPSAV	408
ECAKIII	*L*ALI*ND*SKACA*GKEV*GVLEFPNIRMI CYGA*SHRLCFLV*G*D**QVQKLSHNL**	449

Fig. 2. Comparison of the amino acid sequences of (a) purine PRT and (b) aspartokinase. TFGPT, purine PRT from *T. flavus*; XGPRT, xanthine-guanine PRT from *E. coli*; human HPRT, hypoxanthine PRT; TFAK, aspartokinase from *T. flavus*; CGAK, aspartokinase from *C. glutamicum*; BSAKII, aspartokinase II from *B. subtilis*; ECAKIII, aspartokinase III from *E. coli*. Asterisks denote amino acids identical to those of *T. flavus* enzymes.

Expression of *gpt* in *E. coli*

To detect the enzyme activity encoded by *gpt*, we constructed plasmid pPRT101, which allowed the expression of *gpt* under the control of the *lac* promoter on pUC19. A crude extract prepared from *E. coli* JM105 harbouring pPRT101 was analysed by SDS-PAGE. As shown in Fig. 3, a heat-stable protein with a molecular mass of 17 kDa, which was in good agreement with the molecular mass deduced from the nucleotide sequence of the *gpt* gene, was detected in the crude extract prepared from the *E. coli* transformant, while no such heat-resistant protein was found when the crude extract from *E. coli* without the plasmid was similarly analysed.

Then the PRT activity in the heat-treated extract of *E. coli* harbouring pPRT101 was examined. Since heat-treatment of *E. coli* extracts at 70 °C for 30 min completely inactivated the native PRT (data not shown), the heated crude extract was used for the analysis of *Thermus* purine PRT activity. The reaction was carried out using adenine, hypoxanthine, xanthine or guanine as the substrate (Fig. 4). Conversion of the purine to the corresponding purine nucleotide monophosphate was observed when hypoxanthine, xanthine or guanine were used as the substrates, but not when adenine was used. The specific activities of this PRT with guanine [22.0 U (mg protein)⁻¹], hypoxanthine [19.7 U (mg protein)⁻¹] and xanthine [23.9 U (mg protein)⁻¹] were very similar.

Expression of *askAB* in *E. coli*

For expression of the *askAB* genes in *E. coli*, we constructed two plasmids, pAKT101 and pAKT102, both of which allowed the expression of the *askAB* genes under the control of the *lac* promoter on pUC18. *E. coli* cells carrying pAKT101 or pAKT102 were harvested and fractionated into soluble and insoluble fractions by centrifugation, and the soluble fractions were subjected to heat-treatment at 70 °C for 30 min. When each fraction was analysed by SDS-PAGE, a heat-stable protein of 43 kDa, which corresponds to the molecular mass of the α -subunit of the aspartokinase from *T. flavus*, was found mainly in the soluble fractions of both transformants (Fig. 5). However, a 17 kDa protein, which corresponded to the β -subunit, was not found in any fraction prepared from *E. coli* harbouring pAKT101, even when the fractions were heated to remove heat-labile proteins from the *E. coli* host. This result suggests that, in *E. coli* harbouring pAKT101, only the α -subunit, and not the β -subunit, was translated from the intact *askAB* genes. However, *E. coli* harbouring pAKT102 containing an additional copy of the β -subunit gene just downstream of the original *askAB* operon produced a heat-stable protein of 17 kDa. When the aspartokinase activities were measured with these two heat-treated preparations, the specific activities conferred by pAKT101 and pAKT102 were 21.5 U (mg protein)⁻¹ and 12.7 U (mg protein)⁻¹, respectively, which seemed to be proportional to the

Fig. 3. Production of purine PRT from *T. flavus* in *E. coli*. Lane 1, supernatant of the extract of *E. coli* JM105 grown with IPTG; lane 2, supernatant of the extract of *E. coli* JM105 harbouring pPRT101 grown with IPTG; lanes 3 and 4, same as lanes 1 and 2, respectively, but supernatants were heated at 70 °C to denature heat-labile proteins. The purine PRT directed by pPRT101 is indicated by an arrowhead. Molecular mass markers (lane 5) are: phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α -lactalbumin (14 kDa).

amounts of the α -subunit of aspartokinase produced (see Fig. 5). No difference in the thermostability or the temperature-activity-profile of aspartokinase activities was observed between these two preparations (Fig. 6). Both aspartokinase preparations had activity which was stable up to 80 °C, and each had a temperature-activity profile with an optimum temperature of 75 °C.

We next examined the effects of lysine and threonine on the enzyme activity, because the aspartokinases from *C. glutamicum* and *B. subtilis* are inhibited by the end-products (lysine plus threonine and lysine only, respectively) of the branched amino acid biosynthesis pathway, starting from aspartate through feedback inhibition (Tosaka *et al.*, 1983; Moir & Paulus, 1977). As shown in Table 1, the aspartokinase activities in both extracts were decreased by the addition of threonine, but were not significantly decreased by lysine. When lysine was added to the reaction

Fig. 4. Time-course for the formation of nucleotide monophosphates with (a) adenine, hypoxanthine or xanthine as substrate, and (b) guanine as substrate. ○, Xanthosine monophosphate; □, inosine monophosphate; △, adenosine monophosphate; ◇, guanosine monophosphate; ●, xanthine; ■, hypoxanthine; ▲, adenine; ◆, guanosine.

mixtures simultaneously with threonine, no further decrease in the enzyme activity was observed. Although the aspartokinase activity in both crude extracts was modulated only by threonine, the sensitivity to threonine of the two preparations was different. Addition of threonine to the extract from *E. coli* harbouring pAKT102 caused a marked decrease in the activity (5–10% of the original activity remaining), whereas addition of threonine had a smaller effect on the activity in the aspartokinase preparation of *E. coli* harbouring pAKT101 (about 30% remaining).

DISCUSSION

This study together with our previous study has revealed the architecture of two operons (*askAB-gpt* and *scsAB-mdh*) of *T. flavus*. The aspartokinase genes of *C. glutamicum* and *Mycobacterium smegmatis* were shown to be

Fig. 5. Production of aspartokinase from *T. flavus* in *E. coli*. Lane 1, molecular mass markers; lanes 2–4, *E. coli* JM105 cultured with IPTG as the control; lanes 5–7, *E. coli* JM105 harbouring pAKT102 cultured in the presence of IPTG; lanes 8–10, *E. coli* JM105 harbouring pAKT101 cultured in the presence of IPTG; lanes 2, 5 and 8, supernatants of the sonicates of the *E. coli* cells; lanes 3, 6 and 9, precipitates of the sonicates of the *E. coli* cells; lanes 4, 7 and 10, supernatant of the sonicates after heat-treatment at 70 °C for 30 min. The α - and β -subunits of aspartokinase are arrowed.

adjacent to the gene encoding aspartate semialdehyde dehydrogenase, which catalyses the conversion of aspartylphosphate, formed by the aspartokinase, to aspartate semialdehyde, and were therefore assumed to be co-transcribed as a single mRNA (Kalinowski *et al.*, 1990; Cirillo *et al.*, 1994). In *E. coli* possessing three aspartokinases, two of the three aspartokinase genes are included in gene clusters for biosynthesis of methionine and threonine (Theze & Saint Girons, 1974; Duchange *et al.*, 1983). However, the aspartokinase II of *B. subtilis* is translated from a monocistronic mRNA (Chen *et al.*, 1989). In *T. flavus*, the aspartokinase genes probably form an operon with the gene encoding purine PRT, because the *askAB* genes are close to the purine PRT gene and share two bases. Thus the arrangement around the aspartokinase gene of *T. flavus* is totally different from those of other bacteria.

The promoter region of the aspartokinase II gene of *B. subtilis* contains a short region capable of encoding a lysine-rich peptide, which overlaps with the region of several dyad symmetries to function as an attenuator element controlling the expression of the aspartokinase gene(s) in response to the availability of lysine (Lu *et al.*, 1992). No such short reading frame was present in the upstream region of *askA* of *T. flavus*. This observation may suggest the presence of some other regulatory mechanism for the expression of the aspartokinase genes. It is also of interest to reveal how the expression of this operon is regulated, because it comprises the genes responsible for two independent biosynthetic pathways: aspartokinase is responsible for amino acid biosynthesis

and purine PRT is responsible for salvage synthesis of nucleotides. Transcriptional analysis of this operon is necessary to clarify the regulatory mechanism for its expression.

Aspartokinase catalyses the first step of the branched biosynthetic pathway for lysine, threonine, isoleucine and methionine, and is known to be regulated by the end-metabolites through feedback inhibition. For example, the activity of aspartokinase II of *B. subtilis* is regulated by lysine, while the aspartokinase activity of *C. glutamicum* was concertedly inhibited by lysine plus threonine. In the case of the aspartokinase of *T. flavus*, the activity was inhibited only by threonine. In spite of the relatively high sequence similarity, the amino acid residues of aspartokinase, which are responsible for modulation of the enzyme activity via end-product inhibition, are still unknown. Site-directed mutagenesis based on the sequence similarity along with determination of three-dimensional structure will be required to clarify the mechanism for controlling the enzyme activity.

Two expression plasmids for the aspartokinase from *T. flavus* were constructed, one, pAKT101, with the original *askAB* genes downstream of the *lac* promoter and the other, pAKT102, having a structure similar to that of pAKT101 but with an additional copy of the *askB* gene downstream of the *askA* genes. Both constructs directed the production of the α -subunit, but only pAKT102 gave the detectable production of the β -subunit in *E. coli*. This suggests that a potential RBS for the β -subunit, located in the middle of the *askA* gene, did not function efficiently for the translation of the β -subunit in *E. coli*, and that the

Fig. 6. Temperature-activity profile (a) and thermostability (b) of aspartokinase from *T. flavus*. ●, Aspartokinase prepared from *E. coli* harbouring pAKT102; ○, aspartokinase prepared from *E. coli* harbouring pAKT101. Thermostability was determined by measuring residual activity after heat-treatment at indicated temperatures for 30 min. Control activities were 6.6×10^{-2} U for aspartokinase prepared from *E. coli* harbouring pAKT102 and 2.6×10^{-2} U for aspartokinase prepared from *E. coli* harbouring pAKT101, respectively.

Table 1. Feedback inhibition of aspartokinase from *T. flavus*

Amino acid (1 mM)		Relative aspartokinase activity (%)	
Lysine	Threonine	pAKT101	pAKT102
—	—	100*	100†
+	—	90 ± 3	90 ± 5
—	+	27 ± 4	6 ± 1
+	+	28 ± 3	9 ± 2

* Control activity was 2.6×10^{-2} U.

† Control activity was 6.6×10^{-2} U.

β -subunit produced in *E. coli* carrying pAKT102 was mostly translated from the transcript derived from the additional *askB* gene connected downstream of the *askAB* genes. These two aspartokinase preparations had almost the same thermostability and temperature-activity-profile. Furthermore, both aspartokinase preparations

showed sensitivity to threonine. This suggests that the β -subunit of the aspartokinase is not necessary for the primary function of the enzyme. Until now, the role of the β -subunit had not been clarified. Chen & Paulus (1988) suggested no functional role for the β -subunit of aspartokinase II from *B. subtilis* either in enzyme activity or in feedback inhibition using an *E. coli* expression system which was artificially designed to produce only the α -subunit. However, Kalinowski *et al.* (1990) suggested a functional contribution of the β -subunit; they observed that introduction of the mutated β -subunit gene from aminoethylcysteine-resistant *C. glutamicum* into a wild-type *C. glutamicum* strain caused partial resistance of the aspartokinase to this compound. In this study the aspartokinase preparation containing the β -subunit had higher sensitivity to threonine, compared with the preparation without a detectable amount of the β -subunit. This suggests a possible role of the β -subunit in modulating the *Thermus* aspartokinase activity. From these observations, we assume that threonine may bind to the β -subunit (and also to the COOH-terminal portion of the α -subunit) for the modulation of the enzyme activity, and that the end-product inhibition by threonine is more effective when the enzyme is in the form of a hetero-oligomer (possibly an $\alpha_2\beta_2$ tetramer).

ACKNOWLEDGEMENTS

We are grateful to A. Iida (Tokyo Research Laboratories, Kyowa Hakko Kogyo Co.) for his advice on the measurement of the PRT activity.

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Received 13 October 1994; revised 16 January 1995; accepted 31 January 1995.