THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 269, No. 39, Issue of September 30, pp. 24304–24309, 1994
Printed in U.S.A.

Relationship of Protein Structure of Isoleucyl-tRNA Synthetase with Pseudomonic Acid Resistance of *Escherichia coli*

A PROPOSED MODE OF ACTION OF PSEUDOMONIC ACID AS AN INHIBITOR OF ISOLEUCYL-tRNA SYNTHETASE*

(Received for publication, May 23, 1994, and in revised form, June 28, 1994)

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To elucidate the mode of action of pseudomonic acid, we have compared the deduced amino acid sequences of isoleucyl-tRNA synthetases (IleRS) from wild-type *Escherichia coli* strain MC4100, a pseudomonic acid-resistant mutant (strain PS102) of MC4100, and a pseudomonic acid-producing strain, *Pseudomonas fluorescens*. Compared with the wild-type enzyme, the deduced amino acid sequence of *E. coli* mutant *ileS* gene in strain PS102 shows a single amino acid substitution of leucine for phenylalanine at residue 594 of the IleRS. This mutational alteration in IleRS of an *E. coli* pseudomonic acid-resistant mutant resides in a region of the enzyme in close proximity to one of the consensus sequences of class I aminoacyl-tRNA synthetases, the KMSKS sequence between residues 602 and 606 of the *E. coli* IleRS. DNA sequence of the cloned *ileS* gene predicts that the *P. fluorescens* IleRS consists of 943 amino acids with 54% identity with the *E. coli* IleRS. The *P. fluorescens ileS* gene and the wild type and PS102 alleles of *E. coli ileS* were cloned into an expression vector, pEXPCR, and the sensitivities of *E. coli* DH5α cells harboring each of these plasmids were compared. The cells harboring the *P. fluorescens ileS* were found to be most resistant to pseudomonic acid, while the transformants expressing the PS102 IleRS were more resistant than those containing the wild-type *E. coli* IleRS. IleRS purified from the wild-type *E. coli* was specifically cleaved by trypsin between Lys^{605} and Ser^{606} in the region of $K^{602}MSKS^{606}$. The protection of the IleRS from the trypsin digestion was found with pseudomonic acid or ATP, but not with isoleucine or $tRNA_1^{Ile}$. Based on these results, we propose that pseudomonic acid binds to IleRS in the vicinity of the KMSKS sequence that is an ATP-binding subsite, and that pseudomonic acid is a bifunctional inhibitor with characteristics of both isoleucine and ATP, for example, an analog of isoleucyladenylate.

Pseudomonic acid A (hereafter referred to as pseudomonic acid) is the antibacterial agent produced by *Pseudomonas fluorescens* NCIB 10586 (Fuller *et al.*, 1971). Pseudomonic acid selectively inhibits isoleucyl-tRNA synthetase (IleRS)[1] (Hughes and Mellows, 1978, 1980). This striking specificity in the mode of action places pseudomonic acid into an unusual class of the antibacterial agents (Parenti, 1987; Bryan, 1988; Neu, 1992). The chemical structure of pseudomonic acid is shown in Fig. 1 (Chain and Mellows, 1977; Snider and Phillips, 1982; Snider *et al.*, 1983). It has been proposed that the terminus of the side chain containing the epoxide moiety of the pseudomonic acid (Fig. 1, *Part A*), having the same carbon skeleton as L-isoleucine, competes for the single isoleucine-binding site on the enzyme as an isoleucine analog (Hughes and Mellows, 1978). Consequently, it inhibits IleRS competitively with respect to isoleucine (Hughes and Mellows, 1978; Chain and Mellows, 1974, 1977; Alexander, 1978).

Part B of pseudomonic acid, however, differs greatly from the structure of L-isoleucine and is indispensable for the inhibition of IleRS by pseudomonic acid (Chain and Mellows, 1974, 1977; Alexander *et al.*, 1978; Klein *et al.*, 1989). The precise mechanism of action of pseudomonic acid remains to be elucidated. To investigate the mode of action of pseudomonic acid, we have undertaken the present study by comparing the primary structures of IleRS from wild-type *Escherichia coli* and a spontaneous pseudomonic acid-resistant mutant as well as that from *P. fluorescens*, the pseudomonic acid-producing bacterium that is intrinsically resistant to this antibiotic. We have also studied the interaction of pseudomonic acid with *E. coli* IleRS as measured by protection of IleRS from trypsin digestion by pseudomonic acid. In the present study, we demonstrate that the wild-type *E. coli* IleRS is specifically cleaved by trypsin between Lys^{605} and Ser^{606}, and that this cleavage by trypsin is prevented by pseudomonic acid or ATP, but not by isoleucine or $tRNA_1^{Ile}$. In addition, the pseudomonic acid-resistant mutant IleRS contains a single amino acid substitution of Leu^{594} for Phe^{594}. These results suggest that pseudomonic acid binds to IleRS in the vicinity of the consensus sequence $K^{602}MSKS^{606}$, which is part of the ATP-binding site. We propose that pseudomonic acid is a bifunctional inhibitor that interacts with IleRS as an analog of both isoleucine and ATP.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—*E. coli* strains used in the present study included DH5α (*supE44, lacU169* (ϕ80 *lacZ* M15) *hsdR17 recA endA1 gyrA96 thi1 relA1*, Life Technologies, Inc.), P90A5 derivative Ts331 (F⁻ *argG ileS*^{ts} *lac thi*), a gift of Dr. Leif Isaksson, Department of Microbiology, University of Uppsala (Isaksson *et al.*, 1977), and MC4100 (F⁻ *lacU169 araD139 rpsL150 thi flbB5301 deoC7 ptsF25 relA1*). *P. fluorescens* strain NCIB 10586 was obtained from the National Collec-

* This work was supported by National Institutes of Health Grant GM28811 (to H. C. W.) and annual funds for research from the Ministry of Education of Japan (to M. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X80132.

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[1] The abbreviations used are: IleRS, isoleucyl-tRNA synthetase; 3-letter amino acid abbreviation with the suffix RS, aminoacyl-tRNA synthetase; PCR, polymerase chain reaction.

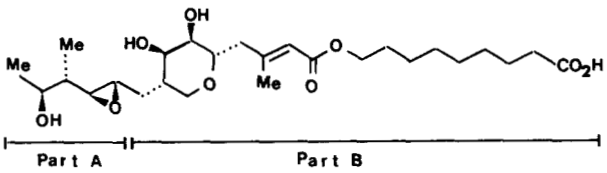

FIG. 1. **Chemical structure of pseudomonic acid A, a potent inhibitor of *E. coli* isoleucyl-tRNA synthetase.** The terminus of the side chain containing the epoxide moiety (*Part A*) possesses the same carbon skeleton as L-isoleucine (Hughes *et al.*, 1980; Alexander *et al.*, 1978)

tions of Industrial and Marine Bacteria. Plasmid pBROC128 was kindly provided by SmithKline Beecham Pharmaceuticals (Isaki *et al.*, 1990b). Plasmid pUC19 (Yanisch-Perron *et al.*, 1985) was purchased from Takara Shuzo Co. Ltd.

Culture Media—Escherichia coli cells were grown in LB or SOB medium (Sambrook *et al.*, 1989). *P. fluorescens* cells were grown at 25 °C in culture media (pH 7.0) containing (per liter) 10 g of Bacto-tryptone, 10 g of Bacto-proteose peptone No.3, 1.5 g of dipotassium phosphate, and 1.5 g of magnesium sulfate.

Materials—The sources of materials used in the present study were as follows. *E. coli* $tRNA_1^{Ile}$ was from Subriden RNA; restriction enzymes were from New England Biolabs. T4 DNA ligase, calf intestine alkaline phosphatase, and bacteriophage T4 DNA ligase were from Toyobo Biochemicals, and Klenow fragment of *E. coli* DNA polymerase I was from Takara Shuzo Co. DNA sequencing kits (the Sequenase version 2.0 labeled dCTP edition, the 7-deaza-dGTP kit) were from U. S. Biochemical Corp., and BcaBest dideoxy sequencing kit was from Takara Biochemicals. *Thermus aquaticus* DNA polymerase was from Perkin-Elmer Corp.; dATP, dGTP, dCTP, and dTTP were from Pharmacia Biotech Inc. Radiochemicals [α-^{32}P]dCTP (800 Ci/mmol) and [U-^{14}C]isoleucine (324 Ci/mol) were from DuPont NEN, and isopropyl-1-thio-β-D-galactopyranoside and 5-bromo-4-chloro-3-indolyl-β-D-galactoside were from Sigma. Pseudomonic acid was a gift from SmithKline Beecham Pharmaceuticals (Betchworth, Surrey). DNA oligonucleotide primers, used for DNA sequencing and polymerase chain reaction, were prepared at the Center for Genetic Research, Nagoya University or purchased from Integrated DNA Technologies, Inc.

DNA Sequencing—DNA sequences were determined from both strands, according to the instructions in DNA sequencing kit, using double-stranded plasmid DNA.

Assay for Isoleucyl-tRNA Synthetase—Aminoacylation of tRNA was assayed as described previously (Kawakami *et al.*, 1985). Protein concentration was estimated by the modified Lowry method (Bensadoun and Weinstein, 1976) using bovine serum albumin as standard.

Partial Digestion of Isoleucyl-tRNA Synthetase by Trypsin and Analyses of Amino-terminal Amino Acid Sequences of Tryptic Fragments—Wild-type IleRS of *E. coli* was purified as described previously (Kawakami *et al.*, 1985). 21.6 μg (0.19 nmole) was preincubated with pseudomonic acid (final concentrations with the addition of 0.5 μl of pseudomonic acid dissolved in dimethyl sulfoxide were 0–20 mM) or each of IleRS substrates (final concentrations for isoleucine, ATP, or $tRNA_1^{Ile}$ were 0–50 mM, 0–100 mM, or 0–15 μM, respectively) in 42.5 μl of 0.1 M Tris-HCl buffer (pH 7.5) containing 2 mM dithiothreitol and 25% (v/v) glycerol. After a 20-min preincubation on ice, 0.5 μl of tosylamino phenylethyl chloromethyl ketone-treated trypsin (800 μg/ml in 0.01 N HCl, 10 mM $CaCl_2$) was added. After a 24-h incubation at 0 °C, the reaction was stopped by the addition of 0.5 μl of 80 mM phenylmethylsulfonyl fluoride, and the tryptic digests were fractionated on a 10% SDS-polyacrylamide gel. For analyses of amino-terminal amino acid sequences of tryptic fragments, 40 μg of IleRS was digested with trypsin, and the reaction products were fractionated on a 10% SDS-polyacrylamide gel and transferred to a Millipore Immobilon P membrane (LeGendre and Matsudaira, 1989). Amino acid sequences were determined with an Applied Biosystem Model 477A protein sequencing system, or a Shimazu Model PSQ-1 protein sequencer.

Isolation of Pseudomonic Acid-resistant Mutant Strain PS102—Strain PS102 was isolated as a spontaneous mutant of strain MC4100 by plating MC4100 cells on LB media containing 20 μg of pseudomonic acid/ml. P1 transduction experiments (Miller, 1972) confirmed that the mutation conferring pseudomonic acid resistance is linked to *ileS*.

Construction of Plasmid pEXPCR—We have converted plasmid pUC19 into an expression vector for PCR products as follows: (i) the intrinsic *Nde*I site in pUC19 was eliminated; (ii) the upstream sequence CTG, followed by the initiation codon (ATG) of the *lacZ'*, was converted

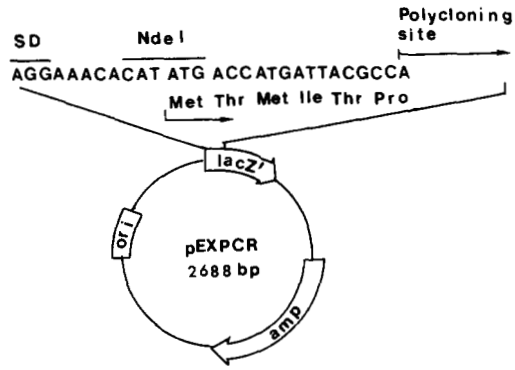

FIG. 2. **The structure of an expression vector (pEXPCR) for the cloning of PCR products.** Any open reading frame beginning at the methionine codon and ending at the stop codon amplified by PCR using specifically designed primers can be inserted between the *Nde*I site and one of the polycloning site (in the order of *Hind*III-*Sph*I-*Pst*I-*Sal*I-*Xba*I-*Bam*HI-*Kpn*I-*Sac*I-*Eco*RI sites) in pEXPCR. For details see "Experimental Procedures."

to the CAT sequence so that a new *Nde*I site (CAT-ATG) was created; (iii) the original polycloning site of pUC19 was preserved; and (iv) the distance between the Shine-Dalgarno sequence and the initiation codon was kept the same as in pUC19. To accomplish these goals, pUC19 DNA was digested with *Nde*I, and the protruding ends of the DNA were filled with Klenow fragment of *E. coli* DNA polymerase 1. Subsequently, the blunt-ended DNA was self-ligated with bacteriophage T4 DNA ligase. The initiation codon for the amino-terminal f-Met in *lacZ'* in this plasmid (called pUCRNde) was converted into a new *Nde*I site by the method of inverse PCR mutagenesis (Clackson *et al.*, 1991). pUCRNde DNA was amplified with a pair of *Nde*I site-inserting primers to generate linear PCR products: 5'-GAGCTTGAAGCAT(ATG)TGTTTCCTGTGTGAAATTGTTATCCGCTC-3' and 5'-TATATCCCAG(CAT)ATGCGCCAAGCTTGCATGCCTG-3' (the *Nde*I sites are in italics, and the initiation codons of methionine in both strands are in parentheses). The PCR products were digested with *Nde*I, and the linear DNA was self-ligated to give a circular vector, pEXPCR. DNA sequence analysis of the amplified DNA demonstrated that this vector was constructed as designed (Fig. 2). The *Nde*I site was inserted at the initiation codon of *lacZ'* so that the pEXPCR vector would express *lacZ'* gene in the presence of isopropyl-1-thio-β-D-galactopyranoside *in vivo*. As an expression vector for PCR products, DNA fragments to be inserted into one of the polycloning sites of the vector were PCR amplified with primers designed to start with the *Nde*I site containing the initiation codon.

Insertions of ileS Alleles into Expression Vector pEXPCR—Plasmids pPS102, pECMC8Z, and pPFNB7 expressing *ileS* alleles from *E. coli* strain PS102, MC4100, and *P. fluorescens*, respectively, were constructed as follows: (i) the coding region of *ileS* was PCR amplified with a pair of the *Nde*I site- and the stop codon-tagging primers using *E. coli* PS102, MC4100, and plasmid pBROC128 as templates; the primers used were 5'-GGAACCGAGAATCAT(ATG)AGTGACTATAAATAACCCTG-3' and 5'-CAGATCGATTAAGCTT(TCA)GGCAAACTTACG-3' for the *E. coli ileS* alleles and 5'-GAAGAGCCTCAT(ATG)ACCGAC-3' and 5'-ACTGGGATCC(TTA)GGCATAGTGGCGAACCTCG-3' for *P. fluorescens ileS* gene, respectively (the restriction enzyme sites are in italics, and the initiation codons or stop codes on the reverse strands are in parentheses). Twenty-five cycles of polymerase chain reactions were performed as follows: 1 min at 94 °C, 1 min at 50 °C, and 2 min at 70 °C; (ii) the extra nucleotides in the PCR products were removed by double digestion so that the structural gene cassettes starting precisely at the initiating f-Met codon and ending at the stop codon were prepared; (iii) the *ileS* gene cassette was ligated with a linear form of pEXPCR obtained by the double digestion at the initiation code (ATG) and the polycloning site of the *lacZ'* gene.

Complementation Studies—E. coli strains Ts331 or DH5α cells were transformed with pEXPCR recombinants containing a series of *ileS* alleles, and the transformants were scored for complementation of the temperature-sensitive phenotypes of mutant Ts331 and for the increased resistance to pseudomonic acid as compared with DH5α. To test for the complementation, the Ts331 transformants were grown at 42 °C on LB agar plates containing ampicillin (50 μg/ml). The growth of the transformants in SOB medium containing pseudomonic acid (0 or 40 μg/ml) and ampicillin (50 μg/ml) was measured based on OD at 600 nm.

TABLE I
Comparison of the deduced amino acid sequences of the ileS genes from E. coli MC4100 and K12

Position (residue no.)	Amino acid sequence	
	MC4100	K 12[a]
243–264	TPWTLPANRAISIAPDFDYALV	RRGLCLPTAQSLLHQISTMRWW
300–301	EL	DV
587	R	C
637	E	Q
724	G	V
738	A	P
740–743	ADSV	RTVW
787	F	L
867–870	GAT	DRRY

[a] The deduced amino acid sequence of IleRS from *E. coli* K12 is given in Webster *et al.* (1984).

RESULTS

Identification of the Mutational Alteration in the ileS Gene of the Pseudomonic Acid-resistant E. Coli Mutant PS102—The *ileS* alleles from the wild-type MC4100 and its spontaneous pseudomonic acid-resistant mutant strain PS102 were PCR amplified and cloned into pEXPCR to generate pPS102 and pECMC8Z, respectively, as described in under "Experimental Procedures." The entire *ileS* coding region in both plasmids was sequenced in both directions. Only one base pair difference between these two *ileS* alleles was found, CTT (Leu^{594}) and TTT (Phe^{594}) for the mutant (pSP102) and the wild-type (pECMC8Z) *ileS* genes, respectively. In addition, the Phe to Leu alteration at residue 594 of the mutant IleRS was confirmed by direct sequencing of PCR product using *E. coli* PS102 DNA as the template in PCR amplification. It is interesting that the mutated site (residue 594) is in the vicinity of a consensus sequence of KMSKS, residues 602–606.

The sequence of the wild-type *ileS* gene determined in the present study is in good agreement with that reported for the *E. coli* strain K12 (Webster *et al.*, 1984), with differences at 37 amino acids, as shown in Table I. It is most likely that the observed differences of 37 amino acid residues in IleRS from these two strains are due to strain differences; such a difference between *E. coli* K12 and MRE600 has been noted previously (Webster *et al.*, 1984).

DNA Sequence of P. fluorescens ileS Gene—Previous work reported that *P. fluorescens ileS* gene is present on plasmid pBROC128 in the order of *X-ileS-lsp-orf149-orf316* (Isaki *et al.*, 1990b) as was found in *E. coli* (Miller *et al.*, 1987) and *Enterobacter aerogenes* (Isaki *et al.*, 1990a). Consequently, we have determined the sequence of the *P. fluorescens ileS* gene in pBROC128. The DNA and deduced amino acid sequence of *P. fluorescens ileS* are shown in Fig. 3. The reading frame of the *ileS* gene consists of 2,829 base pairs encoding 943 amino acids. *P. fluorescens* IleRS exhibits 54% identity of amino acid sequence with the *E. coli* IleRS (Webster *et al.*, 1984), 31% with the yeast IleRS (English *et al.*, 1987), 35% with the *Methanobacterium thermoautotrophicum* IleRS (Jenal *et al.*, 1991), and 31% with the ciliated protozoan *Tetrahymena thermophila* IleRS (Csank and Martindale, 1992).

The two consensus sequences of class I aminoacyl-tRNA synthetases are found in *P. fluorescens* IleRS: HIGH (residues 65–68) and KMSKS (residues 608–612). The PYVPGWDCHGL sequence (residues 90–100) may be a binding site for isoleucine since the same sequence in *E. coli* IleRS has been shown to be involved in isoleucine binding (Clarke *et al.*, 1988; Burbaum *et al.*, 1990). Pseudomonic acid is a competitive inhibitor of *E. coli* B or *P. fluorescens* IleRS with respect to isoleucine (Hughes and Mellows, 1978, 1980; Hughes *et al.*, 1980). Therefore, the pseudomonic acid-binding site and the isoleucine-binding site on IleRS may overlap. Beause the K_i of pseudomonic acid of *P. fluorescens* IleRS is 10^6 times (Hughes *et al.*, 1980) that of *E. coli* IleRS and because both of these enzymes contain the PYVPGWDCHGL sequence as the isoleucine-binding site, an additional sequence is most likely involved in the strong binding of pseudomonic acid to the *E. coli* enzyme.

Comparison of Pseudomonic Acid Resistance Conferred by Various IleRS Alleles—DNA sequence analysis has revealed a single amino acid substitution of Leu for Phe at residue 594 in IleRS encoded by plasmid pPS102 as compared with pECMC8Z. To ascertain whether this single amino acid alteration is responsible for the increased pseudomonic acid resistance in mutant strain PS102 and whether the *P. fluorescens ileS* gene alone is responsible for the extreme resistance of *P. fluorescens* toward pseudomonic acid, we have compared the effects of pseudomonic acid on the growth of DH5α harboring pECMC8Z, pPS102, or pPFNB7.

E. coli strain Ts331 cells harboring pPS102 (*E. coli* mutant *ileS* gene), pECMC8Z (*E. coli* wild-type *ileS* gene), and pPFNB7 (*P. fluorescens ileS* gene) were able to grow on LB agar containing ampicillin (50 μg/ml) at the nonpermissive temperature of 42 °C. These results suggest that each of the *ileS* alleles is expressed *in vivo* to produce sufficient IleRS and complement the temperature-sensitive mutant allele in strain TS331. Overproduction of the IleRS in each of the transformants was also clearly demonstrated by SDS-polyacrylamide gel electrophoresis (data not shown). While each of these three plasmids complemented the IleRS deficiency in Ts331 at 42 °C, they differed in the degree of pseudomonic acid resistance conferred on the host cells. DH5α cells harboring pPS102 were able to grow in the presence of pseudomonic acid (40 μg/ml), while the cells harboring pECMC8Z failed to grow under the same condition (Fig. 4). Since the wild type and the mutant ileS alleles are in the same plasmid construction and are expressed by the same P_{lac} in the vector, the possibility that the difference in pseudomonic acid resistance between DH5α(pPS102) and DH5α(pECMC8Z) results from a difference in the plasmid copy number or the promoter strength is excluded. The results suggest that the change of phenylalanine to leucine at residue 594 of the IleRS results in the increased resistance of *E. coli* IleRS to pseudomonic acid. Plasmid pPFNB7 (containing *P. fluorescens ileS* gene) conferred even far greater pseudomonic acid resistance on DH5α cells than pPS102. Since the *ileS* gene on pPFNB7 is obtained from the pseudomonic acid-producing strain, these results suggest that the IleRS from *P. fluorescens* are intrinsically extremely resistant to pseudomonic acid.

While *E. coli* wild-type IleRS with Phe^{594} is pseudomonic acid-sensitive, *E. coli* IleRS with Leu^{594} is pseudomonic acid-resistant. This result suggests that Phe^{594} of the *E. coli* IleRS may be part of the pseudomonic acid-binding site. On the other hand, the T^{597}HGFTVDETGRKMSKSLK^{614} sequence of the *P. fluorescens* IleRS is 78% identical to the T^{591}HGFTVDGQGRKMKSIG^{608} of *E. coli* IleRS, with Phe^{600} of *P. fluorescens* IleRS corresponding to Phe^{594} of the wild-type *E.*

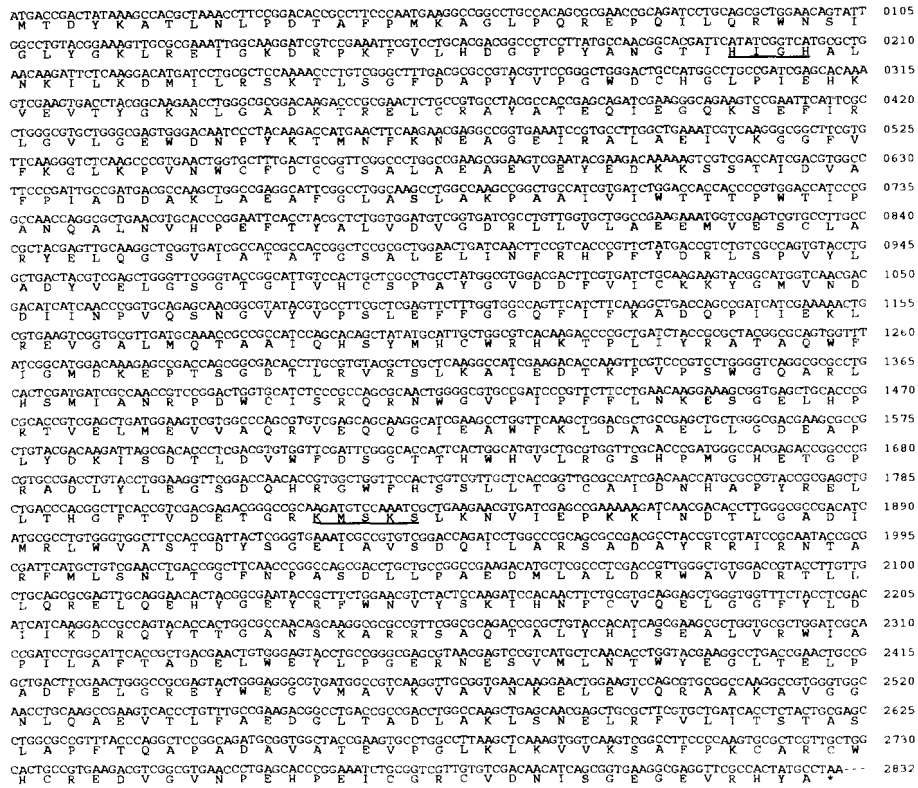

FIG. 3. **The DNA sequence of *P. fluorescens ileS* and the deduced amino acid sequence.**

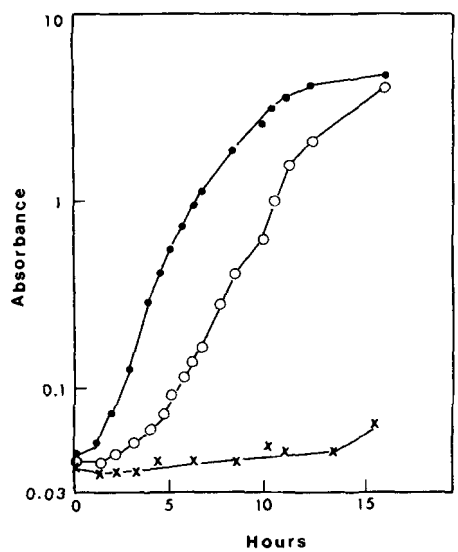

FIG. 4. **Growth of *E. coli* DH5α cells transformed with various alleles of *ileS* in the presence of pseudomonic acid.** Strain DH5α harboring pECMC8Z (*E. coli* wild-type *ileS* gene), pPS102 (*E. coli* mutant *ileS* gene), or pPFN7 (*P. fluorescens ileS* gene), were grown at 37 °C in SOB medium containing 50 μg/ml ampicillin and 40 μg/ml pseudomonic acid. Culture turbidity was monitored as $A_{600\ nm}$ with a light path of 1 cm. ×, pECMC8Z (*E. coli* wild-type *ileS* gene); ○, pPS102 (*E. coli* mutant *ileS* gene); ●, pPFNB7 (*P. fluorescens ileS* gene).

coli IleRS. While *P. fluorescens* IleRS with Phe^{600} is pseudomonic acid-resistant, *E. coli* wild-type IleRS with Phe^{594} is pseudomonic acid-sensitive.

Specific Cleavage of IleRS by Trypsin and Protection by pseudomonic acid or ATP—The partial digestion of the *E. coli* wild-type IleRS with trypsin yielded two fragments. As shown in Fig. 5, SDS-polyacrylamide gel electrophoresis analysis of the tryptic digests revealed three bands: 114 kDa for *band a*, 73 kDa for *band b*, and 39 kDa for *band c*. The amino-terminal amino acid sequences of uncleaved IleRS, fragments *b* and *c* were found to be SDYKSTLNLP, SDYKSTLNLP, and SIGNTVSPQD, respectively. It can be concluded, therefore, that a specific cleavage by trypsin of IleRS occurred at Lys^{605}-Ser^{606} in the G^{598}QGRKMSKSIGNTVSPQD^{615}. The sequence data were also consistent with the apparent M_r of the tryptic fragments in SDS gel.

Considering the fact that the PS102 allele of *ileS* is altered at the site close to the KMSKS sequence and the possibility that the residue 594 may be involved in pseudomonic acid binding to the IleRS, we examined the protection of IleRS from trypsin cleavage by pseudomonic acid in the wild-type IleRS. Fig. 5*C* shows that pseudomonic acid protected IleRS from the specific tryptic cleavage at the KMSKS sequence in a concentration-dependent manner (0–20 μM). Similar protection was observed with ATP (Fig. 5*A*) but not with isoleucine (Fig. 5*B*) or $tRNA_1^{Ile}$ (Fig. 5*D*). These results suggest that pseudomonic acid binds to IleRS in the vicinity of Phe^{594} extending to the KMSKS consensus sequence. The amount of pseudomonic acid that protected IleRS against the tryptic digestion was 3 orders of magnitude lower than that of ATP. This large difference most likely suggests that pseudomonic acid binds to *E. coli* IleRS with greater affinity and avidity than ATP does. In addition, it has been found that, as an ATP analog, pseudomonic acid competitively inhibits *E. coli* IleRS (Table II).

DISCUSSION

Based on their primary structures, aminoacyl-tRNA synthetases are classified into two groups. Class I possesses the HIGH and the KMSKS sequences or their homologs (GlnRS, TyrRS, MetRS, GluRS, ArgRS, ValRS, IleRS, LeuRS, TrpRS, CysRS) (Burbaum *et al.*, 1990; Eriani *et al.*, 1990; Cusack *et al.*, 1990; Eriani *et al.*, 1991; Hou *et al.*, 1991), and Class II lacks both HIGH and KMSKS motifs (ProRS, SerRS, ThrRS, AspRS, AsnRS, HisRS, LysRS, PheRS, GlyRS, AlaRS) (Eriani *et al.*, 1990).

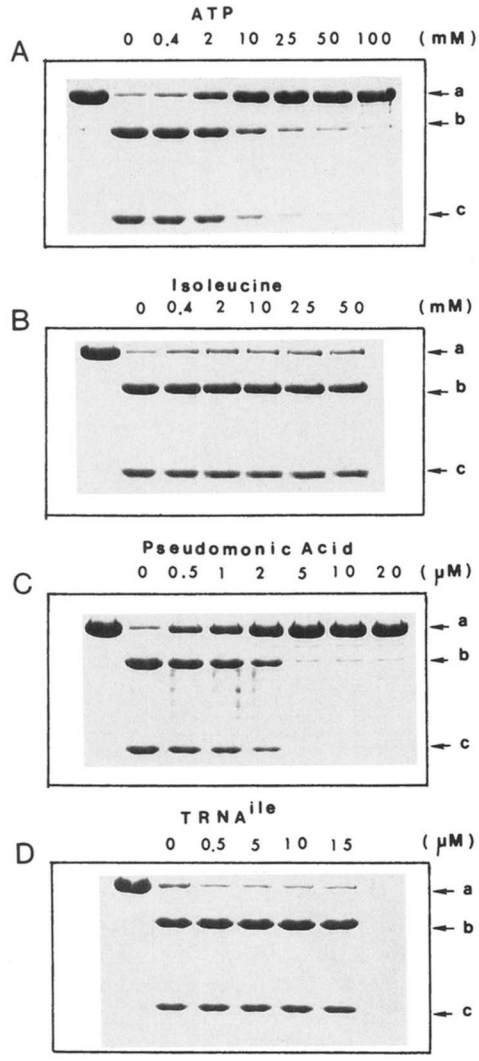

FIG. 5. **The effects of the IleRS substrates or pseudomonic acid on tryptic digestion of *E. coli* isoleucyl-tRNA synthetase.** For details, see "Experimental Procedures." The digests were separated on a 10% SDS-polyacrylamide gel. Tryptic digestion was performed in various concentrations of ATP, L-isoleucine, pseudomonic acid, or $tRNA_1^{Ile}$. *Panel A*, ATP; *panel B*, L-isoleucine; *panel C*, pseudomonic acid; *panel D*, $tRNA_1^{Ile}$. *Arrows* indicate the positions of bands *a*, *b*, and *c*, corresponding to the apparent molecular masses of 114, 73, and 39 kDa, respectively. The first *lanes* on the *left* in *all panels* represent the undigested samples.

TABLE II

Kinetic parameters of E. coli wild type, mutant, and P. fluorescens isoleucyl-tRNA synthetases, pH 7.5, 37 °C

The inhibition constants, K_i, of pseudomonic acid with respect to ATP and K_m values for ATP were estimated from Dixon plots and reciprocal plots in the aminoacylation of tRNA, respectively.

Source of Ile-tRNA synthetase	K_m	K_i
	mM	*nM*
E. coli MC4100	0.30	2.3
E. coli PS102	0.14	16.7
P. fluorescens NCIB 10586	0.08	2,500

In the case of *Bacillus stearothermophilus* TyrRS, the HIGH sequence is shown to be involved in ATP binding as well as in the tyrosyladenylate formation, and the first and second lysine reisidues of the KFGKT sequence (the KMSKS-homolog) interact with the PP_i moiety of ATP (Fersht, 1987; Fersht *et al.*, 1988). In the case of *E. coli* MetRS, the second lysine residue of the KMSKS sequence was identified by affinity labeling experiments as the binding site for the 3′ CCA terminus of tRNA (Hountondji and Blanquet, 1985; Hountondji *et al.*, 1986). The two consensus sequences, HLGH (a homolog of HIGH sequence) and KMSKS, form part of the ATP binding domain with the second lysine of the KMSKS sequence presumably lying near the γ-phosphate of ATP (Brunie *et al.*, 1990, Perona *et al.*, 1991). The KMSKS sequence has also been implicated to be the pyrophosphate subsite and the catalytic center (Mechulam *et al.*, 1991).

We have shown that out of 61 lysine residues in *E. coli* IleRS sequence, Lys^{605} alone is preferentially cleaved by trypsin. This suggests that the second Lys^{605} of $K^{602}MSKS^{606}$ is exposed near the surface of the enzyme in its three-dimensional structure. In view of the facts that (i) the specific cleavage of IleRS by trypsin at Lys^{605} is protected by pseudomonic acid and by ATP; (ii) the pseudomonic acid-resistant *E. coli* mutant IleRS is altered at the site 594 in close proximity to the $K^{602}MSKS^{606}$ sequence; and (iii) pseudomonic acid competitively inhibits IleRS with respect to ATP (Table II), the simplest explanation is that the pseudomonic acid-binding site(s) and ATP-binding site(s) are overlapped in the vicinity of the $K^{602}MSKS^{606}$ region. Alternatively, the protective effects of pseudomonic acid or ATP against the tryptic cleavage of IleRS could result from the allosteric effect of pseudomonic acid or ATP, which renders the KMSKS sequence inaccessible to trypsin. Likewise, a conformational change due to the mutational alteration at Phe^{594} could result in the reduced binding of pseudomonic acid to the mutant enzyme. According to our working hypothesis, *P. fluorescens* IleRS does not bind pseudomonic acid effectively due to the lack of subsite essential for pseudomonic acid binding or due to steric hindrance. Unfortunately, *P. fluorescens* IleRS sequence did not provide any useful clue for the structure (domain and sequence) of pseudomonic acid-binding site(s).

Analyses of the UV-irradiated IleRS·ATP complex revealed that the seventh amino acid from the carboxyl terminus is one of the ATP-binding sites (Yue and Schimmel, 1977; Webster *et al.*, 1989). Kinetic analyses of *E. coli* IleRS showed that pseudomonic acid was a competitive inhibitor for isoleucine (Hughes and Mellows, 1980). Growth of *E. coli* cells is inhibited by pseudomonic acid, and this inhibition is reversed by the addition of isoleucine (Hughes and Mellows, 1978). These observation and the recognition that part A of pseudomonic acid (Fig. 1) has the same carbon skeleton as that of L-isoleucine (Hughes and Mellows, 1980) have led to the postulate that pseudomonic acid inhibits IleRS as an analog of isoleucine (Hughes and Mellows, 1980). On the basis that the Gly^{94} to Arg^{94} mutation of the *ileS* gene results in an increase in the K_m for isoleucine, it has been postulated that the $P^{90}YVPGWDCHLeu^{100}$ sequence of the IleRS, downstream from the consensus sequence of $H^{65}IGH^{68}$ is related to binding of isoleucine (Clarke *et al.*, 1988; Burbaum *et al.*, 1990). In addition to Gly^{94} of IleRS, it has been found that Phe^{570} contributes to the formation of the isoleucine-binding site (Schmidt and Schimmel, 1994). These sites may be, therefore, related to binding site for isoleucine-like part A of pseudomonic acid.

Part B of pseudomonic acid is essential for the inhibition of *E. coli* IleRS by pseudomonic acid as well (Klein *et al.*, 1989). This suggests that in addition to the putative isoleucine-binding site (for isoleucine and part A of pseudomonic acid) in IleRS, additional site(s) participate in the binding of part B. Pseudomonic acid protects *E. coli* IleRS from the cleavage by trypsin at Lys^{605}-Ser^{606}, a site involved in ATP binding. This specific protection by pseudomonic acid is presumably due to the binding of part B of pseudomonic acid to the enzyme. Just as the isoleucine-like part A of pseudomonic acid binds IleRS in the vicinity of the PYVPGWCHGL sequence, part B of pseudomonic acid binds IleRS in the vicinity of the KMSKS region.

L-Isoleucinyladenylate, the synthetic compound condensed from L-isoleucinol (amino alcohol) and AMP, is a strong inhibitor of *E. coli* IleRS with a K_i value of 7.4 nM with respect to isoleucine (Cassio *et al.*, 1967). The K_i value of L-isoleucinol is 3 orders of magnitude higher than that of L-isoleucinyladenylate. This clearly suggests that the adenosine monophosphate moiety participates in the binding of this inhibitor to the IleRS (Cassio *et al.*, 1967). It is not surprising that isoleucinyladenylate is a structural analog of isoleucyladenylate. We propose that pseudomonic acid is an inhibitor of *E. coli* IleRS possessing characteristic features of both an isoleucine analog and an ATP analog, for example, an analog of L-isoleucyladenylate. The K_i value of L-isoleucinyladenylate is of the same order of magnitude as that of pseudomonic acid for *E. coli* wild-type IleRS with respect to isoleucine (Hughes and Mellows, 1978) and ATP. The proposed mechanism of the mode of action of pseudomonic acid may be fruitfully exploited in designing new antibiotics.

Acknowledgments—We thank Dr. Masahiro Sugiura of the Center for Genetic Research, Nagoya University, for providing DNA primers and Philippa Burbidge and Dr. Martin Burnham of SmithKline Beecham Pharmaceuticals for the generous gift of pseudomonic acid.

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