Natural Variation of Tyrosyl-tRNA Synthetase and Comparison with Engineered Mutants†

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Received October 31, 1985; Revised Manuscript Received January 15, 1986

ABSTRACT: We report the cloning and sequence analysis of the gene for the tyrosyl-tRNA synthetase from *Bacillus caldotenax* and properties of the gene product. The amino acid sequence of the tyrosyl-tRNA synthetase was found to be 99% homologous with the corresponding enzyme from *B. stearothermophilus*, with only four amino acid differences. Two of these natural variations were found to involve active site residues of the enzyme and correspond to mutations that have been engineered previously in vitro. One, Thr-51 → Ala-51, produced a more active enzyme, possessing a higher value of k_{cat}/K_M for ATP. Position 51 is a "hot spot" in the tyrosyl-tRNA synthetase, differing in enzymes derived from *Escherichia coli*, *B. stearothermophilus*, and *B. caldotenax*. The other, His-48 → Asn-48, is found to be a neutral mutation but is in one of the rare regions that are conserved with other aminoacyl-tRNA synthetases. The equivalence of histidine and asparagine at position 48 extends the homology in this region to more enzymes. These residues, His-Ile-Gly-His, and now His-Ile-Gly-Asn, form part of the binding site for ATP in the transition state of the reaction. Although *B. caldotenax* is an obligate thermophile with an optimal growth temperature of 80 °C, as much as 20 °C above the growth optima of strains of *Bacillus stearothermophilus*, its tyrosyl-tRNA synthetase has an identical thermal stability in vitro to that from *B. stearothermophilus*.

Tyrosyl-tRNA synthetase (TyrTS) from *Bacillus stearothermophilus* has been extensively studied both structurally and kinetically. X-ray crystallographic analysis of TyrTS at 0.3-nm resolution has been published (Bhat et al., 1982), and a subsequent refinement to a nominal 0.21 nm has recently been obtained (Blow & Brick, 1985). The crystal structure of the enzyme-bound tyrosyl adenylate complex has also been solved (Rubin & Blow, 1981). This information, combined with data from site-directed mutagenesis, yields a direct knowledge of the interactions of the enzyme with its substrates. Amino acid residues predicted to be involved in the binding of ATP have been extensively studied by site-directed mutagenesis of the cloned TyrTS gene (Winter et al., 1982; Wilkinson et al., 1983, 1984; Carter et al., 1984; Fersht et al., 1984, 1985a; Lowe et al., 1985).

In general, the replacement of residues that form hydrogen bonds with substrates produces enzymes of lower activity. The one exception is the replacement of residues at position 51, a position close to the ring oxygen of ATP. In the enzyme from *B. stearothermophilus*, this residue was found to be a threonine whereas in *Escherichia coli* this position is occupied by proline which cannot hydrogen bond with the ATP. The mutation of Thr-51 to alanine, cysteine, or proline produces a family of enzymes that bind ATP increasingly more tightly in the transition state for the formation of the tyrosyl adenylate (Fersht et al., 1985b). As a complementary approach to engineering mutant enzymes in vitro, we are also examining the primary structures of TyrTSs from other species of bacteria. This approach will reveal the natural variation of residues of interest such as position 51. Furthermore, this approach may prove to be relevant to enzyme thermostability. The factors governing protein folding and stability are relatively unknown. The comparison of amino acid sequences of the TyrTS from various mesophilic and thermophilic bacteria may therefore indicate structures that contribute to thermostability. Subsequently, these structures may be probed by site-specific mutagenesis.

† This work was funded by the MRC of the U.K.

We report herein the cloning of the TyrTS gene from *Bacillus caldotenax*, an obligate thermophile with an optimal growth temperature of 80 °C (Heinen & Heinen, 1972).

Experimental Procedures

Enzymes and Reagents. Restriction endonucleases, T4 DNA polymerase, T4 DNA ligase and *Escherichia coli* DNA polymerase I (Klenow fragment) were purchased from New England Biolabs, Bethesda Research Laboratories, Cambridge Biotechnology Limited or Amersham U.K. [α-^{32}P]dATP (specific radioactivity >400 Ci/mmol) was purchased from Amersham U.K.

Preparation of B. caldotenax DNA. High molecular weight DNA from *B. caldotenax* cell paste (a gift from Professor A. Atkinson) was prepared essentially as described by Marmur (1961). The cell paste was resuspended in 150 mM NaCl, 25 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl),[1] pH 8.0, and 2 mM EDTA and treated with lysozyme (2 mg/mL) for 20 min at 37 °C. $NaDodSO_4$ was added to 1% (w/v) and the suspension heated at 65 °C for 10 min. Sodium perchlorate was added to 1 M, and the solution was cooled to room temperature. After three extractions with chloroform–isoamyl alcohol (24:1 v/v), the aqueous phase was precipitated with ethanol and resuspended in 0.1 × SSC buffer (SSC buffer: 15 mM trisodium citrate and 150 mM NaCl, pH 7.0). Residual RNA and protein were removed by digestion at 37 °C with ribonuclease A and then with Pronase. The DNA was reextracted with chloroform–isoamyl alcohol and precipitated with ethanol. Finally, DNA was redissolved in 10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA.

Molecular Cloning of TyrTS Gene from B. caldotenax. B. caldotenax DNA (10 μg) was partially digested with restriction endonucleases *Eco*RI (10 units) and *Bam*HI (10 units) at 37 °C for 15 min. After extraction with phenol and precipitation with ethanol, the DNA was cloned into the

[1] Abbreviations: Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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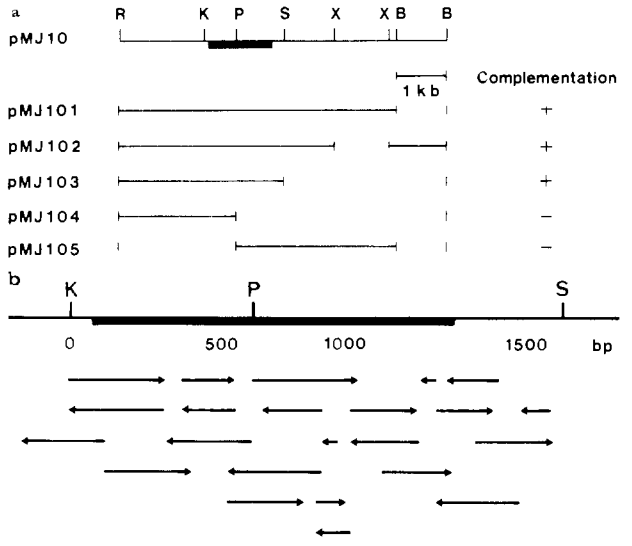

FIGURE 1: (a) Restriction map of the 6.5 kilobase pair insert of pMJ10 (top) and various constructs of this plasmid. The portion of the insert contained in each recombinant plasmid is indicated by a thin solid line. The ability of recombinant plasmids to complement *E. coli*, strain 565cN, and permit growth at 42 °C is indicated by a plus sign. The dimensions of the TyrTS gene coding region are indicated by the bold line beneath pMJ10. Restriction enzyme sites shown are the following: R, *Eco*RI; K, *Kpn*I; P, *Pst*I; S, *Sal*I; X, *Xho*I; B, *Bam*HI. (b) Strategy for sequencing the TyrTS gene. The arrows indicate the direction and extent of DNA sequenced in each of the M13 clones.

plasmid vector pUC8 (Vieira & Messing, 1982) and digested with the same enzymes as described by Maniatis et al. (1982). The ligation mixture was transfected into competent *E. coli* 565cN cells (temperature sensitive in TyrTS) as described by Barker (1982). Approximately 5000 recombinant clones were obtained. Cells containing plasmids capable of complementing the temperature-sensitive TyrTS mutation were selected on ampicillin agar plates at 42 °C. Four recombinants were obtained, and one, pMJ10, was chosen for further study.

pMJ10 was found to contain a 6.5 kilobase pair insert (see Figure 1). Deletion derivatives of pMJ10 were constructed utilizing the *Bam*HI, *Sal*I, and *Pst*I polylinker sequence in pUC8. Deletions pMJ101, pMJ102, pMJ103, and pMJ104 were constructed by digestion of pMJ10 with the respective restriction enzyme and isolation of the DNA fragment containing pUC vector sequences from agarose gels as described by Dretzen et al. (1981). Following self-ligation and transfection into *E. coli* 565cN, the plasmids were tested for complementation at 32 and 42 °C. pMJ105 was constructed by cloning the indicated *Pst*I–*Bam*HI fragment (Figure 1) into *Pst*I/*Bam*HI digested pUC9.

DNA Sequence Analysis. The "dideoxy" DNA sequencing method of Sanger et al. (1977) was used as described by Bankier and Barrell (1983). The *Kpn*I–*Sal*I DNA fragment was isolated and subdigested with the following enzymes: *Pst*I, *Rsa*I, *Taq*I, *Ava*I, *Hin*fI, and *Hin*cII singly or in combination. These resulting subfragments were cloned into suitable M13mp8 and M13mp9 vectors (Messing & Vieira, 1982) and sequenced by the dideoxy method (Sanger et al., 1977; Bankier & Barrell, 1983). Sequence data were assembled using the DBUTIL program (Staden, 1980).

Enzyme Purification and Kinetic Assays. Both *B. caldotenax* and *B. stearothermophilus* TyrTSs were prepared from genes expressed in *E. coli* and purified to electrophoretic homogeneity as described by Wilkinson et al. (1982) and Lowe et al. (1985). The thermal stability of TyrTS in 144 mM Tris-HCl (pH 7.8 at 20 °C), 0.1 mM phenylmethanesulfonyl fluoride, and 10 mM 2-mercaptoethanol at 70 °C was determined by taking at timed intervals 5-μL samples and rapidly cooling these samples by mixing with 45 μL of active-site cocktail (Wilkinson et al., 1983) at 0 °C. Active-site titration and kinetic measurement of pyrophosphate exchange were performed as described previously (Jakes & Fersht, 1975; Wilkinson et al., 1983).

Table I: Kinetics of Aminoacylation of $tRNA^{Tyr}$ by Tyrosyl-tRNA Synthetases[a]

enzyme	k_{cat} (s^{-1})	K_M(ATP) (mM)	k_{cat}/K_M (s^{-1} M^{-1})
B. stearothermophilus[b]	4.7	2.5	1860
His-48 → Asn-48[c]	4.9	2.1	2333
Thr-51 → Ala-51[d]	4.0	1.2	3200
B. caldotenax (His-48 → Asn-48, Thr-51 → Ala-51)	6.9	2.0	3450

[a] Kinetic constants for variation of ATP were determined in the presence of 0.1 mM tyrosine, at pH 7.78 (144 mM Tris-HCl), and 10 mM $MgCl_2$ (free). [b] Fersht et al. (1984). [c] Lowe et al. (1985). [d] Wilkinson et al. (1984).

RESULTS

Interspecies Homology of Tyrosyl-tRNA Synthetases. A genomic library of *B. caldotenax* DNA was prepared in the plasmid vector pUC8 (Vieira & Messing, 1982), and the gene for TyrTS was selected by complementation of a strain of *Escherichia coli* containing a temperature-sensitive TyrTS (Barker, 1982). One recombinant clone, pMJ10, was found to contain an insert of approximately 6.5 kilobase pairs. The position of the TyrTS gene was mapped by deletion analysis to be between the *Kpn*I and *Sal*I restriction enzyme sites (Figure 1a). Suitable restriction fragments were isolated and subcloned into M13mp8 and M13mp9 vectors (Messing & Vieira, 1982) and sequenced by the chain termination method (Sanger et al., 1977; Bankier & Barrell, 1983). The DNA sequence was determined completely on both strands, and all restriction endonucleases sites used for generating M13 clones were overlapped (Figure 1b). The sequence is presented in Figure 2 and is compared with the sequence of the *B. stearothermophilus* gene and protein as determined by Winter et al. (1983). The comparison revealed only 24 nucleotide base changes in the protein coding region. Nineteen are silent third base codon changes that do not alter the amino acid sequence. The remaining five changes result in four amino acid substitutions (Figure 2). Two of these amino acid changes involve residues known to be at the active site of the enzyme (Asn-48 → His-48 and Ala-51 → Thr-51). The other two changes (Leu-55 → Met-55 and Ala-297 → Thr-297) are far from the active site and unlikely to affect enzyme activity. The two genes are thus 98% homologous and the two enzymes 99% homologous.

Enzymatic Activity. The properties of the TyrTS were investigated after expressing the gene in *E. coli*. The enzyme from *B. caldotenax* has a higher value of k_{cat} and a lower value of K_M for ATP in aminoacylation than the TyrTS from *B. stearothermophilus* (Table I). Both enzymes have very similar variations of rate with tyrosine concentration. The enzyme from *B. caldotenax* is therefore more active at all concentrations of ATP. The enzyme from *B. caldotenax* also has a higher value of k_{cat} in pyrophosphate exchange (Table II).

The two amino acid changes in the active site have been engineered individually into the gene for *B. stearothermophilus* TyrTS by site-directed mutagenesis. The Thr-51 → Ala-51 mutation (Wilkinson et al., 1984) has been shown to yield an enzyme with a decreased value of K_M for ATP (in aminoacylation) and a slightly lower value of k_{cat}. Mutation of His-48 to Asn yields an enzyme with kinetic parameters almost

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ACCGCGACGAACACTCGCCCCTTCATCACGGGCGGGTGTTTTCTTTTGCATGACATTTAT   60

                M  D  L  L  A  E  L  Q  W  R  G  L  V  N  Q    15
AGAGAGGTGAAGGACATGGATTTGCTTGCGGAATTGCAATGGCGCGGGCTCGTCAACCAA  120
 A

 T  T  D  E  D  G  L  R  K  L  L  N  E  E  R  V  T  L  Y  C    35
ACGACGGATGAAGACGGGTTGCGCAAGCTGTTGAATGAGGAGCGGGTGACGCTCTACTGC  180
                  A     A

                                 H        T           M
 G  F  D  P  T  A  D  S  L  H  I  G  N  L  A  A  I  L  T  L    55
GGGTTTGACCCGACGGCGGACAGTTTGCATATCGGCAACTTGGCCGCTATTTTGACGCTG  240
                                    C        A  C        A

 R  R  F  Q  Q  A  G  H  R  P  I  A  L  V  G  G  A  T  G  L    75
CGCCGCTTCCAGCAGGCGGGGCACCGGCCGATCGCCCTAGTCGGCGGAGCGACGGGGTTG  300
                                 T

 I  G  D  P  S  G  K  K  S  E  R  T  L  N  A  K  E  T  V  E    95
ATCGGTGACCCGAGCGGGAAAAAAAGCGAGCGCACGCTCAATGCGAAAGAAACCGTCGAG  360
    C                                    C

 A  W  S  A  R  I  K  E  Q  L  G  R  F  L  D  F  E  A  D  G   115
GCATGGAGCGCGCGTATTAAAGAACAGCTCGGCCGTTTTTTAGATTTTGAAGCGGACGGC  420
                   C

 N  P  A  K  I  K  N  N  Y  D  W  I  G  P  L  D  V  I  T  F   135
AATCCGGCGAAAATCAAAAACAACTACGACTGGATCGGGCCGCTGGATGTCATTACGTTT  480

 L  R  D  V  G  K  H  F  S  V  N  Y  M  M  A  K  E  S  V  Q   155
TTGCGCGATGTGGGCAAGCATTTCAGCGTCAACTACATGATGGCGAAAGAGTCGGTGCAA  540
       C  C                                             T

 S  R  I  E  T  G  I  S  F  T  E  F  S  Y  M  M  L  Q  A  Y   175
TCGCGCATCGAGACGGGCATTTCATTTACCGAGTTCAGCTATATGATGCTGCAGGCATAC  600

 D  F  L  R  L  Y  E  T  E  G  C  R  L  Q  I  G  G  S  D  Q   195
GATTTTCTCCGCCTGTACGAGACGGAAGGCTGCCGCCTGCAAATCGGCGGGAGCGACCAA  660
                      A

 W  G  N  I  T  A  G  L  E  L  I  R  K  T  K  G  E  A  R  A   215
TGGGGCAACATCACGGCAGGGCTTGAGCTTATTCGCAAAACGAAAGGCGAGGCGCGGGCG  720

 F  G  L  T  I  P  L  V  T  K  A  D  G  T  K  F  G  K  T  E   235
TTTGGCTTGACGATCCCGCTTGTGACGAAAGCGGACGGCACGAAATTCGGGAAAACGGAA  780

 S  G  T  I  W  L  D  K  E  K  T  S  P  Y  E  F  Y  Q  F  W   255
AGCGGCACGATTTGGCTCGATAAAGAGAAAACATCGCCGTACGAGTTTTACCAGTTTTGG  840
                  C     G

 I  N  T  D  D  R  D  V  I  R  Y  L  K  Y  F  T  F  L  S  K   275
ATCAACACCGACGACCGCGATGTGATCCGCTACTTGAAGTACTTTACGTTCCTGTCAAAA  900
          T           T           T           G

 E  E  I  E  A  L  E  Q  E  L  R  E  A  P  E  K  R  A  A  Q   295
GAGGAAATCGAAGCGCTTGAACAAGAGCTTCGTGAAGCCCCAGAGAAGCGGGCGGCCCAA  960
                                 G

    T
 K  A  L  A  E  E  V  T  K  L  V  H  G  E  E  A  L  R  Q  A   315
AAGGCGCTTGCTGAGGAAGTGACAAAGCTCGTGCACGGCGAAGAGGCGCTCAGGCAAGCG 1020
  A

 I  R  I  S  E  A  L  F  S  G  D  I  A  N  L  T  A  A  E  I   335
ATTCGCATTTCTGAAGCGCTCTTTAGCGGCGACATTGCCAATTTGACAGCGGCGGAAATT 1080

 E  Q  G  F  K  D  V  P  S  F  V  H  E  G  G  D  V  P  L  V   355
GAGCAAGGGTTCAAAGATGTGCCGTCATTCGTTCATGAAGGAGGCGACGTTCCGCTTGTC 1140
                  A

 E  L  L  V  S  A  G  I  S  P  S  K  R  Q  A  R  E  D  I  Q   375
GAGCTGCTCGTTTCTGCCGGCATTTCGCCATCGAAGCGGCAAGCGCGCGAAGACATCCAA 1200
                                 A

 N  G  A  I  Y  V  N  G  E  R  L  Q  D  V  G  A  I  L  T  A   395
AACGGCGCCATTTACGTCAACGGCGAGCGCCTTCAAGACGTCGGAGCCATATTAACGGCT 1260

 E  H  R  L  E  G  R  F  T  V  I  R  R  G  K  K  K  Y  Y  L   415
GAACACCGCCTTGAGGGGCGGTTTACGGTCATCCGCCGCGGGAAGAAAAAGTATTATTTG 1320

 I  R  Y  A  *
ATTCGCTACGCCTAAGAAAAAAGATCCGAAATGCATCGGCATTTCGGATCTTTTTTGCGC 1380

CTTAACGCGAGTAGAACTCGACGATGAGCGCTTCGTTGATTTCCGCCGGCAGTTCGGAAC 1440

GCTCCGGCAAGCGGGTGTACGTCCCTTCCATTTTTTCCGGATTGAACGTCAAGTAATCCG 1500

GAATGTAGTTGTTCGCTTCGAGCGCTTCTTTAATCACTTGCAAGTTGCGCGATTTTTCGC 1560

GAACGGCGATCGTTTGTCCCGGTTTGACTC 1590
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FIGURE 2: Nucleotide sequence of the DNA and derived amino acid sequence. The numbers at the end of each line correspond to the base in the DNA sequence (numbered from the A residue of the *Kpn*I recognition sequence) and the amino acid residue in the protein. The bases below the DNA sequence indicate the differences with the *B. stearothermophilus* gene sequence, and those residues above the amino acid sequence indicate the differences with the *B. stearothermophilus* protein sequence.

identical with those of the wild-type *B. stearothermophilus* enzyme (Lowe et al., 1985). The *B. caldotenax TyrTS* has a similar value of k_{cat}/K_M (ATP variation) to the *B. stearothermophilus* TyrTS(Ala-51) but has a higher value of k_{cat} in aminoacylation. This leads to a higher activity of the enzyme from *B. caldotenax* at cellular concentrations of ATP (Fersht et al., 1985b).

Thermal Stability. The enzymes from *B. stearothermophilus* and *B. caldotenax* have identical thermal stabilities at 70 °C in the standard Tris buffer (pH ~ 6.2; Figure 3). At pH 7.0, in phosphate buffer of ionic strength 0.1, the half-life at 70 °C of both is increased 4-fold to 1 h (data not shown). The identical thermal stabilities is perhaps surprising, considering the difference between the growth temperatures of the two species. The strain of bacteria from which the *B. stearothermophilus* TyrTS gene was isolated, NCA 1503, grows optimally at 58 °C (Atkinson et al., 1979). In contrast, *B. caldotenax* has a growth optimum of 80 °C (Heinen & Heinen, 1972). However, the similarity in thermostability is not surprising in consideration of the amino acid sequence homology of the two TyrTSs. The thermal stability of both enzymes in vivo may well be increased by other factors. For example, we find that enzyme thermostability is increased in vitro by increasing the salt concentration of buffers (unpublished results).

Table II: Kinetics of Pyrophosphate Exchange[d]

enzyme	k_{cat} (s^{-1})	K_M(ATP) (mM)	k_{cat}/K_M (s^{-1} M^{-1})
B. stearothermophilus[a]	7.6	0.9	8400
His-48 → Asn-48[b]	7.9	1.4	5640
Thr-51 → Ala-51[c]	8.6	0.54	15900
B. caldotenax (His-48 → Asn-48, Thr-51 → Ala-51)	15.7	2.0	7850

[a] Fersht et al. (1984). [b] Lowe et al. (1985). [c] Wilkinson et al. (1984). [d] Measurements in the presence of 50 μM tyrosine.

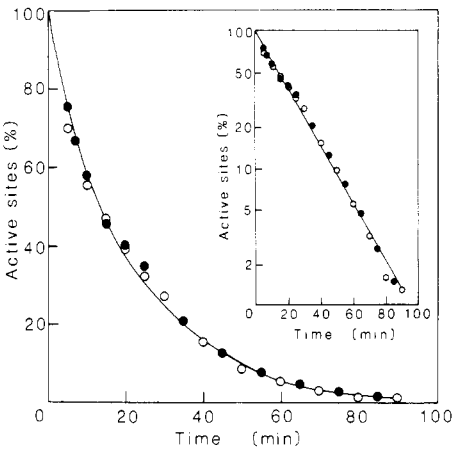

FIGURE 3: Inactivation of *B. caldotenax* and *B. stearothermophilus* TyrTSs at 70 °C. The insert shows a semilogarithmic plot of the data. The solid circles represent data from *B. caldotenax* TyrTS at 7.8 μM, and open circles represent data from *B. stearothermophilus* TyrTS at 5.4 μM.

Discussion

The degree of homology between the genes for TyrTS from *B. stearothermophilus* and *B. caldotenax* suggests that these two species of bacteria are very closely related. The discovery of two variant residues within the active site of the TyrTS is surprising in consideration of the relatedness of the two species of *Bacillus*.

Homologies with Other Aminoacyl-tRNA Synthetases. Although distinct homologies exist at the level of tertiary structure, the aminoacyl-tRNA synthetases are, in general, a vastly divergent family of enzymes with few homologies in primary structure. Therefore, conservative or homologous regions, identified by a comparison of primary sequences, are likely to be important in catalysis and functionally equivalent.

Crystallographic analysis of the *E. coli* methionyl-tRNA synthetase (MetTS) (Zelwer et al, 1982) indicates that it has a similar tertiary structure to the TyrTS, with a characteristic

			45 48	
TyrTS(a)	33	L Y C G F D P T A D S L H I G H L A T I		52 (1)
TyrTS(b)	33	L Y C G F D P T A D S L H I G N L A A I		52 (2)
TyrTS	35	L Y C G F D P T A D S L H L G H L V P L		54 (3)
TrpTS	6	V F S G A Q P S G E L T I G N Y M G A		24 (4)
TrpTS(a)	4	I F S G I Q P S G V I T I G N Y I G A		22 (5)
MetTS	9	V T C A L P Y A N G S I H L G H M L E H		28 (6)
MetTS(c)	200	I T S A L P Y V N N V P H L G N I I G S		219 (7)
IleTS	53	L H D G P P Y A N G S I H I G H S V N K		72 (8)
GlnTS	28	H T R F P P E P N G Y L H I G H A K S I		47 (9)

FIGURE 4: Alignment of the amino acid sequences from the HIGH regions. Unless otherwise indicated, sequences are derived from *E. coli* enzymes. Sequences derived from other origins are (a) *B. stearothermophilus*, (b) *B. caldotenax*, and (c) *Saccharomyces cerevisiae*. The numbers at either end refer to the position of the sequence relative to the amino terminus of the enzyme. The number in parentheses gives the reference to the sequence: (1) Winter et al. (1983); (2) this paper; (3) Barker et al. (1982a); (4) Hall et al. (1982); (5) Winter and Hartley (1977); (6) Barker et al. (1982b); (7) Walter et al. (1983); (8) Webster et al. (1984); (9) Hoben et al. (1982).

nucleotide binding fold topology (Blow et al., 1983).

Figure 4 shows the amino acid homology between the TyrTSs (Barker et al., 1982a; Winter et al., 1983) and the MetTSs (Barker et al., 1982b; Walter et al., 1983) around the residues involved in ATP binding. Also shown are the similar regions from other synthetases (Winter & Hartley, 1977; Hall et al., 1982; Hoben et al., 1982; Webster et al., 1983) all of which contain a "HIGH" (His-Ile-Gly-His) sequence or variant thereof. The HIGH sequence, first identified by Webster et al. (1983), represents the most significant homology identified, to date, between any two aminoacyl-tRNA synthetases.

The substitution of an asparagine for histidine at position 48, in the *B. stearothermophilus* TyrTS, produces an enzyme that is kinetically indistinct from wild type (Table I). Consequently, the sequence "HIGN" (His-Ile-Gly-Asn) and the sequence HIGH may be considered functionally equivalent. It therefore becomes possible to extend the homology in this region to include other enzymes. MetTS from *Saccharomyces cerevisiae* contains an HIGH sequence.

The importance of the HIGH(N) region has become apparent from other studies (Fersht et al., 1984; Leatherbarrow et al., 1985). Histidine or asparagine at position 48 binds ATP. More significantly, His-45 binds the γ-phosphoryl group of ATP in the transition state and is responsible for a considerable factor of the catalytic rate.

The high degree of homology that exists between the TyrTSs and the TrpTSs leads us to speculate that the regions aligned with positions 45–48, in Figure 4, are part of the ATP binding site of the TrpTSs. Although the TrpTSs have an asparagine-48 equivalent, they do not possess a histidine-45 equivalent. However, as alignment of these sequences requires a deletion (at position 43), it is possible that the threonine residue fulfills the role of histidine at position 45.

Lowe et al. (1985) have discussed how asparagine may substitute for histidine in those cases where the δ-nitrogen of the imidazole is involved in hydrogen-bonding interactions but not when the ϵ-nitrogen is involved. Note that as asparagine cannot substitute for histidine at position 45 (Fersht et al., 1984), we would not expect the sequence NIGH(N) to appear in the active sites of other aminoacyl-tRNA synthetases.

Within the active sites of many enzymes there are unique residues that are important for chemical catalysis. TyrTS, on the other hand, appears to utilize many small binding interactions to enhance rate. Catalysis is delocalized around the active site, principally by hydrogen bonding of the substrates to side chains and the polypeptide backbone (Fersht et al., 1986). There does not appear to be the requirement for any one particular catalytic residue at any one site in the protein (e.g., Asp-102, His-57, and Ser-195 of the serine proteases). Thus, the active sites of the aminoacyl-tRNA synthetases need not be identical in sequence to be conserved. There is just the requirement for equivalent hydrogen-bonding sites. Those regions of other aminoacyl-tRNA synthetases that demonstrate a sequence homology to residues 45–48 of the TyrTS are, by inference, responsible for ATP binding. It is noteworthy that the regions of homology exist only in the amino-terminal portions of the bacterial aminoacyl-tRNA synthetases.

Site-directed mutagenesis and primary sequence analysis are complementary approaches to the study of enzymes. Together they may implicate residues or regions that are important in catalysis and, alternatively, those that are dispensible. Where no refined crystal structure exists for an enzyme, as in the case of the TrpTSs, these approaches may provide the only avenue by which the active site can be defined.

ACKNOWLEDGMENTS

We thank Dr. D. G. Barker for the *E. coli* strain 565cN and Professor A. Atkinson for the gift of the *B. caldotenax* cell paste.

Registry No. DNA (*Bacillus caldotenax* tyrosyl-tRNA synthetase gene), 100857-86-1; tyrosyl-tRNA synthetase (*Bacillus caldotenax* reduced), 100857-90-7; tyrosyl-tRNA synthetase, 9023-45-4.

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Internal Thermodynamics of Position 51 Mutants and Natural Variants of Tyrosyl-tRNA Synthetase†

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Received September 26, 1985; Revised Manuscript Received December 5, 1985

ABSTRACT: Natural variation and evolution impose structural changes on an enzyme that can affect the energetics of catalysis. The energy profile of reaction could, in theory, be altered in three distinct ways: uniform binding changes, differential binding changes, and catalysis of elementary steps. Residue threonine-51 of tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* is subject to natural variation, being replaced by alanine and proline in the enzymes from *Bacillus caldotenax* and *Escherichia coli*, respectively. The consequences of this variation on the energetics of formation of tyrosyl adenylate have been investigated by constructing free energy profiles for wild-type and mutant enzymes constructed by introducing these amino acids into the *B. stearothermophilus* enzyme. Mutation of Thr-51 to alanine, proline, and cysteine by site-directed mutagenesis improves the stabilization of the transition state in the formation of tyrosyl adenylate. Most marked is the mutation Thr-51 → Pro-51 which stabilizes the transition state by 2.2 kcal/mol and accelerates the forward rate 20-fold to a level near that of the enzyme from *E. coli*. However, the improved transition-state binding is accompanied by an even greater stabilization of tyrosyl adenylate. This reduces the rate of pyrophosphorolysis of tyrosyl adenylate and/or weakens the binding of pyrophosphate in the reverse reaction, shifting the equilibrium between enzyme-bound reactants greatly in favor of the enzyme–intermediate complex. The more stable mutant enzyme–tyrosyl adenylate complexes have lower rates of aminoacylation, suggesting that mutations which stabilize the intermediate slow down the subsequent transfer of tyrosine from tyrosyl adenylate to tRNA. In contrast, the natural variants have apparently evolved additional mechanisms to bind the transition state preferentially without further stabilizing tyrosyl adenylate. The free energy profiles reveal all three classes of energetic changes on mutation.

The evolution of rates and specificity of enzyme-catalyzed reactions has been the subject of several theoretical studies (Fersht, 1974; Crowley, 1975; Cornish-Bowden, 1976; Albery & Knowles, 1976). It has been proposed that enzymes maximize rates by binding transition states strongly and substrates weakly (Pauling, 1946) and by avoiding disadvantageous accumulation of intermediates (Fersht, 1974). For enzymes following Michaelis–Menten kinetics, this is achieved by improving the specificity constant (k_{cat}/K_M) while maintaining the Michaelis constant (K_M) above the physiological concentration of substrate (Fersht, 1974). In an analysis of enzymes following Briggs–Haldane kinetics, improvements in rate are envisioned to proceed through three major types of structural changes that alter the free energies of enzyme-bound complexes until the rate becomes diffusion controlled (Albery & Knowles, 1976). These steps have been designated "uniform binding", "differential binding", and "catalysis of the elementary steps" according to their distinctive effects on the free energy profile of the catalyzed reaction.

Much of the supportive experimental evidence has centered on the kinetic constants of present-day enzymes. The hypotheses that K_M values tend to match in vivo levels of substrates and that k_{cat}/K_M increases to values expected for macromolecular diffusion are supported by the kinetic properties of several glycolytic enzymes (Fersht, 1985, pp 327–331). The free energy profile for one of these enzymes, triose-

† This work was supported by the MRC of the U.K.
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0006-2960/86/0425-1891$01.50/0