A Single Substitution in the Motif 1 of *Escherichia coli* Lysyl-tRNA Synthetase Induces Cooperativity toward Amino Acid Binding†

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ABSTRACT: The constitutive lysyl-tRNA synthetase (LysRS) of the *Escherichia coli* strain OEL134 differs from the wild-type enzyme by the single substitution of threonine 208 with methionine. *In vitro* study of the isotopic [^{32}P]PP_i−ATP exchange reaction catalyzed by purified T208M LysRS revealed specific features that are not observed with the wild-type LysRS: (i) The steady state of the reaction was reached after a ~1-min lag when the addition of the enzyme was used to initiate the reaction. This lag disappeared upon preincubation of the enzyme with lysine and ATP. (ii) The variation of the steady state rate as a function of the lysine concentration in the assay was sigmoidal (Hill coefficient of 1.65), suggesting cooperativity of lysine binding to this dimeric enzyme. The allosteric behavior of the mutant enzyme was further established by showing that, at low concentrations of lysine, low amounts of cadaverine stimulated T208M LysRS activity. T208A LysRS, in which threonine 208 had been changed into alanine by site-directed mutagenesis, displayed the same properties as T208M LysRS. Remarkably, Thr 208 makes part of the first signature motif of class II aminoacyl-tRNA synthetases, a motif likely to be involved in the dimerization of the enzyme subunits. Therefore, the behavior of the Thr 208 mutants of LysRS supports the idea that the dimerization of class II aminoacyl-tRNA synthetases is important for an efficient structuration of their active site.

Aminoacyl-tRNA synthetases (aaRS)[1] are a key group of enzymes involved in maintaining the fidelity of protein synthesis through the specific esterification of an amino acid (aa) to the 2′- or 3′-hydroxyl group of the 3′-terminal adenosine of the cognate tRNA(s). This reaction usually occurs on a two-step basis, which can be summarized as follows:

$$\text{aaRS} + \text{aa} + \text{ATP} \rightleftharpoons \text{aaRS:(aa}\sim\text{AMP)} + PP_i$$

$$\text{aaRS:(aa}\sim\text{AMP)} + \text{tRNA} \rightleftharpoons \text{aa}\sim\text{tRNA} + \text{aaRS} + \text{AMP}$$

Although they share a common reaction frame, aaRSs are characterized by a large structural diversity, as reflected by differences in amino acid sequences, subunit sizes, and quaternary structures. Attempts to unify the family of aaRSs through structural correlations remained unsuccessful until the sequencing of the full set of the 20 *Escherichia coli* aminoacyl-tRNA synthetases, as well as the recognition of consensus motifs (Webster *et al.*, 1984; Hountondji *et al.*, 1986), allowed the partition into two classes of equal sizes (Eriani *et al.*, 1990). This partition is supported also by the crystal structures of various class I and class II aaRSs (Brick *et al.*, 1989; Rould *et al.*, 1989; Brunie *et al.*, 1990; Cusack *et al.*, 1990; Ruff *et al.*, 1991; Biou *et al.*, 1994; Delarue *et al.*, 1994; Onesti *et al.*, 1995). Class I enzymes display the nucleotide binding fold first described by Rossmann (Rossmann *et al.*, 1974), while class II enzymes display a novel folding topology built around an antiparallel β-sheet (Cusack *et al.*, 1991).

The three consensus motifs which characterize class II enzymes form the core of the protein and part of the dimer interface (Moras, 1992). Motifs 2 and 3 both exhibit a single invariant arginine and a few semi-invariant hydrophobic or glycine residues at key positions. Structural data showed that motif 2 makes up two strands of the antiparallel β-sheet, whereas motif 3 makes another strand. Site-directed mutagenesis of various residues in these two motifs revealed the crucial role of both conserved arginine residues for ATP binding and aminoacyl adenylate formation (Cavarelli *et al.*, 1994; Davis *et al.*, 1994; Lu & Hill, 1994). Other residues in these motifs are involved in the formation of the catalytic site and in the binding of either the amino acid, the ATP, or the tRNA acceptor stem (Cavarelli *et al.*, 1993, 1994; Belrhali *et al.*, 1994; Ibba *et al.*, 1994; Poterszman *et al.*, 1994; Shi *et al.*, 1994; Onesti *et al.*, 1995).

Much less information is available on motif 1. Actually, this sequence consensus has only been precisely identified in the class II synthetases which are homodimeric. In these cases, the only fully conserved residue of motif 1 is a proline. The 3-D structures of five class II enzymes (*E. coli* and *Thermus thermophilus* SerRSs, *Saccharomyces cerivisiae* and *T. thermophilus* AspRSs, and *E. coli* LysRS) show that the peptide containing motif 1 forms an α-helix involved in the association between subunits and a short β-strand interacting through main-chain hydrogen bonds with motif 2. Substitution in yeast AspRS of the conserved proline, located in the short β-strand, by a glycine indicates the importance of this residue for dimerization, and also suggests that the dimeric

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[1] Abbreviations: aaRS, aminoacyl-tRNA synthetase; PP_i, inorganic pyrophosphate; PCR, polymerase chain reaction. Mutations are designated by the wild-type amino acid, followed by the residue position, which is followed by the mutant amino acid (e.g., T208M refers to methionine replacing threonine at position 208).

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nature of AspRS is a prerequisite for its activity (Eriani *et al.*, 1993).

In *E. coli*, two distinct LysRS species occur (Hirshfield *et al.*, 1981). One of them results from the constitutive *lysS* gene, while the other one is the product of the inducible *lysU* gene. The two LysRSs have very close sequences, with 88% identical residues (Lévêque *et al.*, 1990). In addition, with AspRS and AsnRS, LysRS forms a subgroup within the class II family (Cavarelli & Moras, 1993). Previous work showed that a lysine bradytroph *E. coli* strain (OEL134) produced low amounts of LysRS activity and that this low activity was likely to be caused by a mutation in the *lysS* gene (Boy *et al.*, 1976; Hassani *et al.*, 1991). The origin of this defect is confirmed in the present study. The mutant LysRS carries a single substitution in motif 1. Interestingly, the mutated enzyme displays positive cooperativity toward lysine binding. Such behavior is interpreted to result from a perturbation of the dimer interface with a distant effect on the lysine binding sites. This conclusion reinforces the current view that dimerization of class II aaRS subunits is important in maintaining a productive conformation of the two active sites.

MATERIALS AND METHODS

Bacterial Strains. All bacterial strains used through this work were *E. coli* K12 derivatives: JM101TR (*supE thi* Δ*(lac-pro) recA56 srl-300*::Tn*10* F′*(traD36 lacI*^{q} *proAB lacZ*Δ*M15)*) (Hirel *et al.*, 1988), OEL134 (*lys-1102*) (Boy *et al.*, 1976), and PAL3103SΔK (F$^-$ Δ*(lac-pro) gyrA rpoB metB argE*(am) *ara supF* Δ*lysS*::*kan*) (Lévêque *et al.*, 1991).

Recombinant DNA Techniques. Restriction endonucleases and DNA modifying enzymes were purchased from Boehringer, Bethesda Research Laboratory, or Pharmacia. General genetic and cloning techniques were followed as described in Sambrook *et al.* (1989). Genomic DNA was prepared according to Harris-Warrick *et al.* (1976). Oligonucleotides were synthesized by the β-cyanoethyl phosphoramidite method, using an automated Gene Assembler I (Pharmacia), and purified by chromatography on a MonoQ anion exchanger (0.5 × 5 cm, from Pharmacia). DNA sequencing was performed on an automated ALF system (Pharmacia) using the dideoxy chain termination method (Sanger *et al.*, 1980), with either single- or double-stranded DNA template.

Plasmids. Cloning vectors used in this study included plasmids pBluescript M13+KS and M13+SK (Stratagene) and phage M13mp18 (Messing, 1983). Plasmid pXLysKS1 was previously described (Lévêque *et al.*, 1990). It harbors a 3.5-kb fragment carrying the *E. coli* wild-type *lysS* gene.

E. coli tRNA^{Lys} was overexpressed from a synthetic gene built as described previously (Meinnel *et al.*, 1988) and inserted into pBSTNAV. After purification from bulk *E. coli* tRNA by liquid chromatography, 1 A_{260} unit of the overproduced tRNA^{Lys} accepted 1200 pmol of lysine.

In the case of *in vitro* site-directed mutagenesis experiments, a 1-kbp *Pst*I−*Hpa*I fragment containing part of the *lysS* gene was subcloned from pXLysKS1 into M13mp18, to give M13PHlysS. Mutagenesis was carried out on single-stranded DNA of this phage (Sayers *et al.*, 1988). The entire *lysS* gene with the desired mutation was constructed as follows. Plasmid pC1 was obtained by filling in the unique *Hin*dIII and *Sal*I sites of pXLysKS1 and replacing its 1-kb *Pst*I−*Hpa*I fragment by a 0.1-kb linker fragment containing a single *Hin*dIII site. Then, mutated *Pst*I−*Hin*dII fragments from M13PHlysS were inserted between the unique *Pst*I and *Hin*dII sites of pC1 and counter-selected by *Hin*dIII digestion.

Cloning of OEL134 lysS Gene. To clone the *lysS* gene of strain OEL134, a PCR amplification was performed with 25 pmol of each of the two oligonucleotides CATTCTAGACGTTCTTATGTCCTTGATGACTCCCGCATT and CACAAGCTTATTTGCCGGGGTTGTGAGCATAACGTA and 1 μg of OEL134 genomic DNA. Thirty cycles of amplification (94 °C for 1 min, 47 °C for 2 min, and 70 °C for 2 min) were carried out using the Pharmacia-LKB Gene ATAK controller. The amplified fragment (1.7 kb) was purified through agarose gel electrophoresis and recovered by electroelution. After digestion by *Xba*I and *Hin*dIII enzymes, the fragment was inserted into plasmid pBluescript SK.

Construction of Strain PALΔSΔUTR. To study mutant LysRSs, a conditional Δ*lysS* Δ*lysU* strain was constructed by using the gene disruption technique of Hamilton *et al.* (Hamilton *et al.*, 1989). More precisely, a 6-kb *Xho*I−*Sac*I fragment containing the *lysU* gene was inserted between *Sac*I and *Sal*I sites of plasmid pMAK705 (Hamilton *et al.*, 1989). Then, a 1064-b *Sac*II fragment including the promoter and the first 124 codons of the *lysU* gene was deleted, to give pMAKΔU. This plasmid was used to transform the ΔlysS strain PAL3103SΔK. Since the replication of pMAK705 is thermosensitive, the integration of pMAKΔU into the chromosome could be followed by selecting transformants at 42 °C on LB-agar plates containing chloramphenicol. After subsequent growth at 30 °C during about 40 generations, cells which no longer carried the plasmid into their chromosome were identified as chloramphenicol-sensitive colonies at 42 °C. Approximatively half of them were also thermosensitive in the absence of chloramphenicol, suggesting that the *lysU* deletion was carried by the chromosome and that the only functional *lysU* gene was carried by the plasmid. One out of the 40 thermosensitive clones was made deficient in recombination by transducing the *recA56* allele of the Hfr strain JC10240 (Hfr(PO45) *thr-3000 recA56 srl-300*::Tn*10 relA1 ilv-318 spoT1 thi-1 rpsE2300*) (Csonka & Clark, 1980), which gave PALΔSΔUTR.

Enzyme Purification. Wild-type LysRS enzyme and the T208M and T208A mutants were purified from strain PALΔSΔUTR transformed by either pXLysKS1 or the pC1 derivatives harboring the mutant genes. Cells were grown at 37 °C in 2 L of 2× TY medium containing 60 μg/mL of ampicillin and 0.33 mM IPTG. The cultures were harvested by centrifugation during 45 min at 5000*g*, and the pellets were resuspended in a 20 mM Tris-HCl buffer (pH 7.8) containing 10 mM 2-mercaptoethanol and 0.1 mM EDTA. All the following steps were performed at 4 °C. Ultrasonic disruption of the cells, removal of the bulk of nucleic acids by streptomycin sulfate precipitation, and concentration of the proteins by ammonium sulfate precipitation were performed as described previously (Brevet *et al.*, 1995). After dialysis against a 75 mM potassium phosphate buffer (pH 6.75) containing 10 mM 2-mercaptoethanol and 0.1 mM EDTA, the protein sample was applied onto a hydroxylapatite column (2.6 × 18 cm; BioRad). Elution was performed using a linear gradient of potassium phosphate concentration (from 75 to 400 mM, pH 6.75). As previously described for the wild-type LysRS overexpressed from *lysS* (Brevet *et al.*, 1995), the mutant enzymes eluted as two peaks on the hydroxylapatite column. The major peak of activity eluting

at the higher ionic strength (more than 80% of the total activity) was kept for further characterization and concentrated by addition of 70% ammonium sulfate. After centrifugation for 10 min at 10000*g*, the protein pellets were dissolved in a 20 mM Tris-HCl buffer (pH 7.8) containing 10 mM 2-mercaptoethanol and 0.1 mM EDTA, and dialyzed first against this buffer and then against the same buffer containing 60% glycerol. Purified enzymes were stored in glycerol at −20 °C.

Gel Filtration and Protein Electrophoresis. Analytical molecular sieving was conducted on a Sephacryl S300 column (1.1 × 100 cm; Pharmacia) equilibrated in a 20 mM Tris-HCl buffer (pH 7.8) containing 50 mM KCl, 10 mM 2-mercaptoethanol, and 0.1 mM EDTA. *E. coli* ValRS of M_r 108 000 and a truncated monomeric form of *E. coli* MetRS (MetRS547) of M_r 64 000 (Mellot *et al.*, 1989) were added as molecular weight markers to each sample analyzed.

Protein electrophoresis was performed using the Pharmacia Phast System in native or SDS-denaturating conditions, with the molecular weight markers recommended by the supplier. Gels encompassed a homogenous 12.5% polyacrylamide gel and a gradient 8−25% polyacrylamide gel.

Enzymatic Assays. L-Lysine-dependent isotopic [^{32}P]PP_i−ATP exchange was assayed at 28 °C in a reaction mixture containing 20 mM Tris-HCl (pH 7.8), 10 mM 2-mercaptoethanol, 0.1 mM EDTA, and various concentrations of lysine, ATP, and PP_i. The magnesium concentration was systematically adjusted so that it exceeded by 3 mM the sum of the ATP and PP_i concentrations in the reaction mixture. In some experiments, cadaverine (from Aldrich) was included in the reaction mixture.

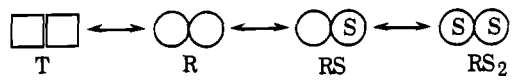
For steady-state measurements, the enzyme was preincubated for 5 min at 28 °C in the presence of all components of the reaction mixture except [^{32}P]PP_i. The reaction was further initiated by the addition of [^{32}P]PP_i and stopped 0−10 min later by the addition of 2.5 mL of a solution containing 50 mM sodium acetate, 100 mM unlabeled PP_i, 0.35% (w/v) perchloric acid, and 0.4% (w/v) norite. The labeled ATP adsorbed on norite was filtered and counted as described before (Blanquet *et al.*, 1974). Pre-steady state kinetics were followed the same way, except that several substrates were omitted in the preincubation mixture and added when the reaction was started.

The $tRNA^{Lys}$ aminoacylation was assayed at 28 °C in a reaction mixture containing 20 mM Tris-HCl (pH 7.8), 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 5 mM $MgCl_2$, 2 mM ATP, 5 μM $tRNA^{Lys}$, and various concentrations of L-[^{3}H]lysine. For steady-state measurements, the enzyme was preincubated for 5 min at 28 °C in the presence of all components of the reaction mixture except $tRNA^{Lys}$. The reaction was further initiated by the addition of $tRNA^{Lys}$ and stopped 0−10 min later by the addition of 2.5 mL of trichloroacetic acid. The precipitated lysyl-$tRNA^{Lys}$ was filtered and counted as described previously (Lawrence *et al.*, 1973).

Enzyme concentrations were measured using a UV absorption coefficient of 0.5 A_{280} units mg^{-1} mL (Brevet *et al.*, 1989), assumed to be identical for wild-type and mutant LysRSs.

Michaelis and cooperative kinetic parameters were derived from iterative nonlinear least-squares fits of the theorical equation (Michaelis, Hill, and Monod−Wyman−Changeux equations) to the experimental values, using the Levenberg−Marquardt algorithm. Confidence limits on the fitted values were obtained by 300 Monte Carlo simulations followed by least-squares fitting, using the experimental standard deviations on individual measurements (Dardel, 1994).

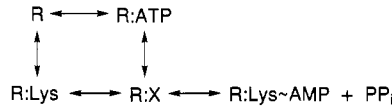
Calculation of Kinetic Constants for Mutant LysRSs. To analyze the allosteric behavior of mutant LysRSs, it was assumed that (i) according to the classical Monod−Wyman−Changeux model, the enzyme occurs as two conformations, R and T; (ii) lysine binds to either of the sites of the R-form with the same intrinsic dissociation constant; and (iii) lysine binding to the T-form is negligible. In diagrammatic form, the reaction scheme may be written



The interconversion between the two forms R and T is characterized by the allosteric constant L_o:

$$L_o = [T]/[R] \quad (1)$$

where [R] and [T] refer to the free concentrations of the R- and T-conformers, respectively. By analogy with other aminoacyl-tRNA synthetases, the ATP−PP_i exchange reaction on one R-subunit can be schematized as follows



In the presence of saturating ATP and PP_i concentrations, the binding of lysine to the R-form can be described by a single apparent dissociation constant K_R:

$$K_R = 2[R^{ATP}_{ATP}][Lys]/[R^{ATP}_X] = [R^{ATP}_X][Lys]/2[R^X_X] \quad (2)$$

where [Lys] is the total concentration of lysine and $[R^B_A]$ represents the concentration of the R-form with substrate A bound to one subunit and substrate B bound to the other subunit.

Finally, the rate of ATP−PP_i exchange is proportional to the concentration of the X-complexed R-form:

$$v_R = \frac{d[[^{32}P]ATP]}{dt} = k_R([R^X_X] + [R^{ATP}_X]/2) \quad (3)$$

where k_R represents the catalytic constant associated with the R dimer.

Equations 1−3 can be used to calculate the steady-state rate of the ATP−PP_i exchange reaction (v_s) as a function of the total concentrations of enzyme ($[E_{tot}]$) and lysine in the assay:

$$v_s = [E_{tot}] \frac{k_R \frac{[Lys]}{K_R}\left(1 + \frac{[Lys]}{K_R}\right)}{L_o + \left(1 + \frac{[Lys]}{K_R}\right)^2} \quad (4)$$

In the presence of the competitive inhibitor cadaverine at a total concentration equal to [Cad], eq 4 becomes:

$$v_s = [E_{tot}] \frac{k_R \frac{[Lys]}{K_R}\left(1 + \frac{[Lys]}{K_R} + \frac{[Cad]}{K_{IR}}\right)}{L_o + \left(1 + \frac{[Lys]}{K_R} + \frac{[Cad]}{K_{IR}}\right)^2} \quad (5)$$

where K_{IR} represents the dissociation constant of cadaverine from its complex with the R-form in the presence of saturating ATP:

$$K_{IR} = 2[R_{ATP}^{ATP}][Cad]/[R_{ATP}^{ATP:Cad}] = [R_X^{ATP}][Cad]/[R_X^{ATP:Cad}]$$

($[R_A^{ATP:Cad}]$ is the concentration of the R-form with substrate A bound to one subunit and ATP and cadaverine bound to the other subunit)

As shown in the Results section, the occurrence of a lag to reach the steady-state kinetics indicates that the transition from the T- toward the R-conformers is very slow compared to the catalytic constant of the reaction. Let k_{L_o} and k_{-L_o} be the rate constants corresponding to the conversion between the T- and R-species:

$$T \underset{k_{-L_o}}{\overset{k_{L_o}}{\rightleftharpoons}} R \leftrightarrow RS \leftrightarrow RS_2$$

The evolution of the T- and R-species is governed by the following equations:

$$\frac{d[T]}{dt} = k_{-L_o}[R] - k_{L_o}[T]$$

$$k_{-L_o}/k_{L_o} = L_o$$

and R, RS, and RS_2 species being in rapid equilibrium:

$$[E_{tot}] = [T] + [R]\left(1 + \frac{[Lys]}{K_R}\right)^2$$

In these conditions, the rate of the ATP−PP_i exchange reaction can be written

$$v_R = v_s - (v_s - v_i)e^{-\lambda t}$$

where v_s is given by eq 4 and where

$$v_i = [E_{tot}] \frac{k_R \frac{[Lys]}{K_R}}{(1 + L_o)\left(1 + \frac{[Lys]}{K_R}\right)} \quad \text{and}$$

$$\lambda = k_{L_o} \frac{L_o + \left(1 + \frac{[Lys]}{K_R}\right)^2}{\left(1 + \frac{[Lys]}{K_R}\right)}$$

Consequently, the concentration of labeled ATP as a function of time is equal to

$$[[^{32}P]ATP] = v_s t - \frac{v_s - v_i}{\lambda}(1 - e^{-\lambda t}) \quad (6)$$

This relationship was used to fit the data obtained under pre-steady-state conditions.

RESULTS

Cloning and Sequencing of the OEL134 lysS Gene. Previous work suggested that the lysine bradytroph strain OEL134 of *E. coli* was mutated in the *lysS* gene encoding constitutive LysRS (Boy *et al.*, 1976; Hassani *et al.*, 1991). To characterize this mutation, a 1.7-kb DNA fragment containing this gene was amplified by standard PCR techniques and cloned into plasmid pBluescript SK. The sequencing of this fragment revealed two point mutations in the *lysS* gene of OEL134. The first one changes the threonine 208, located within motif 1 of LysRS, into a methionine (ACG → ATG). The second one replaces the alanine codon 302 by another alanine codon (GCA → GCG). The sequencing of three independent clones confirmed that these mutations had not been introduced during the PCR amplification.

Purification of the Mutant Enzyme. To obtain the mutant LysRS of OEL134 (T208M LysRS) free of wild-type LysRS, the chromosomal *lysU* gene of a Δ*lysS* strain was disrupted, while the wild-type *lysU* gene was recovered on a thermosensitive plasmid also carrying a chloramphenicol resistance gene. Then, the strain was made deficient for recombination by transducing the *recA56* mutation of strain JC10240. Finally, the resulting strain, PALΔSΔUTR, was transformed by the pBluescript derivative carrying the *lysS* gene of OEL134. All transformants grew at 42 °C and were sensitive to chloramphenicol at this temperature. This behavior suggested that the mutant LysRS protein expressed from a multicopy plasmid was capable of sustaining *E. coli* growth and, therefore, that it was partially active. By a similar procedure, a strain producing the wild-type LysRS enzyme (*lysS* gene product) was obtained by plating at 42 °C PALΔSΔUTR cells transformed by plasmid pXLysKS1. As a control, it was verified that transformation of strain PALΔSΔUTR by plasmid pBluescript did not yield any thermoresistant clone. The latter result indicates that no significant LysRS activity can be expressed from the chromosomal DNA carrying the two deleted Δ*lysS* Δ*lysU* genes.

Purifications of both T208M and wild-type LysRS was carried out from the above strains as detailed in Materials and Methods. Because of the high level of overproduction of the two enzymes, a single chromatographic step on hydroxylapatite was sufficient to yield a protein homogeneous by more than 90% as judged by SDS−PAGE analysis. Routinely, about 40 mg of pure enzyme was obtained per liter of culture.

Cooperative Kinetic Behavior of LysRS T208M. Preliminary studies of the isotopic $[^{32}P]PP_i$−ATP exchange reaction catalyzed by T208M LysRS revealed that the amount of labeled ATP did not increase linearly with the reaction time when the reaction was initiated by the simultaneous addition of ATP (2 mM), lysine (2 mM), and $[^{32}P]PP_i$ (2 mM) to the enzyme. A lag of approximately 1 min occurred before the steady state was reached (Figure 1). This behavior, which contrasted with that of wild-type LysRS, will be discussed further below. In subsequent experiments, unless otherwise stated, mutant or wild-type LysRS was systematically preincubated with lysine and ATP during 5 min, prior to

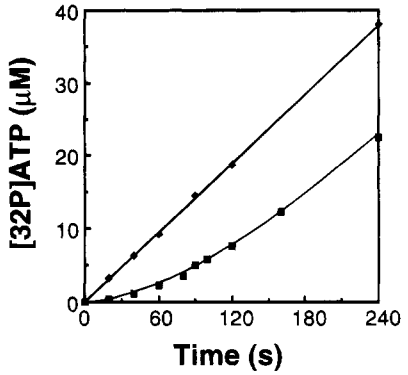


FIGURE 1: Time course of the [^{32}P]PP_i−ATP exchange reaction catalyzed by T208M LysRS. The reaction mixture, buffered with 20 mM Tris-HCl (pH 7.8), contained 2 mM ATP, 2 mM [^{32}P]PP_i, 2 mM lysine, 7 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, and 40 nM T208M LysRS. After a 5-min preincubation of all components of the reaction except enzyme or PP_i, the reaction was started by addition of either enzyme (■) or [^{32}P]PP_i (◆), respectively. After incubation for various times at 28 °C, labeled ATP was adsorbed on charcoal, filtered, and counted as described in Materials and Methods. The continuous curves describing the number of [^{32}P]ATP molecules formed as a function of time were drawn according to the Monod−Wyman−Changeux model described in the text, with the parameters given in Table 2 and a k_{L_o} value of 0.007 s^{-1}.

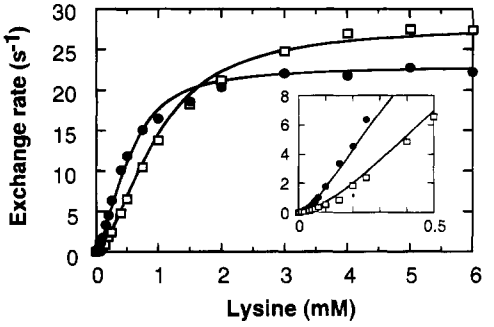


FIGURE 2: Dependence on lysine concentration of the steady-state rate of the ATP−PP_i exchange reaction catalyzed by T208M and T208A LysRSs. The reaction mixture, buffered with 20 mM Tris-HCl (pH 7.8), contained 2 mM ATP, 2 mM [^{32}P]PP_i, 7 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, various concentrations of lysine, and either 40 nM T208M LysRS (□) or 40 nM T208A LysRS (●). All the components of the reaction except PP_i were mixed and preincubated for 5 min at 28 °C prior to the addition of [^{32}P]PP_i, which was used to start the reaction. The continuous curves were drawn according to the Monod−Wyman−Changeux model described in the text, with the parameters given in Table 2. Inset: enlargement of the part of the figure corresponding to lysine concentrations between 0 and 0.5 mM.

the addition of labeled PP_i, which initiated the exchange reaction. In these conditions, the time lag was no longer observed (Figure 1) and the initial rate of the ATP−PP_i exchange proportionally varied with the enzyme concentration in the assay.

When either the ATP or the PP_i concentration was varied at fixed concentrations of the other two ligands (10 mM lysine and 2 mM PP_i, or 10 mM lysine and 2 mM ATP, respectively), the steady-state rates of the ATP−PP_i exchange followed the standard Michaelis equation. The K_m values of the mutant enzyme for ATP and PP_i were respectively 3- and 2-fold higher than those of the wild-type enzyme (Table 1). The corresponding associated k_{cat} values were approximately one-half the values obtained for the wild-type LysRS under the same conditions.

When lysine concentration was varied at saturating ATP and PP_i concentrations (2 mM each), the variation of the steady-state rates did not follow a hyperbolic curve (Figure 2). Data could be satisfactorily fitted to the Hill equation with an interaction coefficient of 1.65 (Table 1), indicative of a positive homotropic effect of the mutant enzyme for lysine binding.

To confirm the cooperative behavior of T208M LysRS with respect to lysine binding, we studied the effect of cadaverine on the rate of the ATP−PP_i exchange reaction. Cadaverine is a chemical analog of lysine lacking the carboxyl group. It behaves as a competitive inhibitor for the binding of lysine to wild-type LysRS (Fromant *et al.*, 1981). As shown in Figure 3, the addition of low concentrations of cadaverine to T208M LysRS stimulated the exchange activity at lysine concentrations below 0.75 mM.

Such an enhancement of the reaction rate by cadaverine is straightforwardly accounted for by the allosteric behavior of the mutant enzyme (Stebbins & Kantrowitz, 1992). Kinetic data with and without cadaverine could be fitted to the Monod−Wyman−Changeux (MWC) model, under the assumptions that (i) the mutant enzyme can exist in two conformational forms, T and R, and (ii) the T-form does not bind either lysine or cadaverine to appreciable extents (see Materials and Methods). The latter hypothesis will be justified below. The activator effect of cadaverine may be explained as follows: When the lysine concentration is undersaturating, the major part of the enzyme population is under the T-form, and the binding of cadaverine will result in the conversion of T-forms into R-forms. Consequently, both the number of high-affinity lysine binding sites and the rate of the exchange reaction will increase. Upon an increase in lysine concentration in the assay, the contribution of cadaverine to the conversion of T- to R-form decreases and the competitive inhibition of cadaverine dominates. Mechanisms based on a preferred kinetic pathway to a ternary

Table 1: Kinetic Constants of the ATP−PP_i Exchange Reaction Catalyzed by Wild-Type, T208M, or T208A LysRS[a]

	Variable substrate					
		ATP[c]			PP_i[d]	
LysRS	lysine,[b] Hill coefficient	Hill coefficient	K_m (μM)	k_{cat} (s^{-1})	Hill coefficient	K_m (μM)
wild-type (LysS)	1.01 ± 0.09	0.91 ± 0.09	174 ± 39	50 ± 6	1.10 ± 0.11	53 ± 7
T208M	1.65 ± 0.09	1.05 ± 0.08	446 ± 27	28 ± 3	1.15 ± 0.10	94 ± 11
T208A	1.55 ± 0.09	0.93 ± 0.16	209 ± 33	23 ± 2	1.12 ± 0.11	67 ± 8

[a] All kinetic measurements were performed at 28 °C in a reaction mixture containing 20 mM Tris-HCl buffer (pH 7.8), 10 mM 2-mercaptoethanol, 0.1 mM EDTA, and a $MgCl_2$ concentration exceeding by 3 mM the sum of ATP and PP_i concentrations in the assay. In all cases, the enzyme was preincubated for 5 min at 28 °C in the presence of all components of the reaction except PP_i. The data were fitted according to the Hill or Michaelis−Menten equation. All of the k_{cat} values were equal within experimental error. For the sake of simplicity, only the value determined when ATP was the variable substrate is shown. [b] 2mM ATP and 2 mM PP_i. [c] 10 mM lysine and 2 mM PP_i. [d] 10 mM lysine and 2 mM ATP.

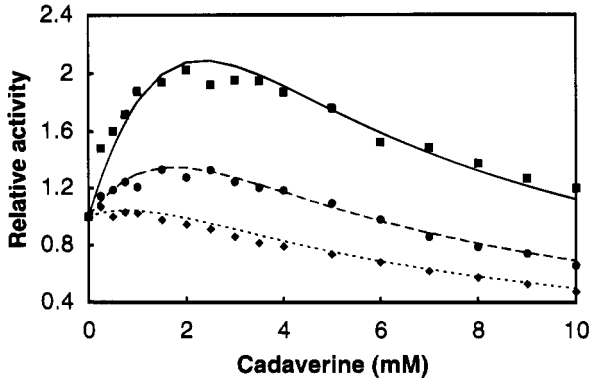


FIGURE 3: Effect of the addition of cadaverine on the steady-state rate of the ATP−PP_i exchange reaction catalyzed by T208M LysRS. The reaction mixture, buffered with 20 mM Tris-HCl (pH 7.8), contained 2 mM ATP, 2 mM [^{32}P]PP_i, 7 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 0.1 μM T208M LysRS, various concentrations of cadaverine, and either 0.15 (■), 0.3 (●), or 0.6 mM lysine (◆). All the components of the reaction except PP_i were mixed and preincubated for 5 min at 28°C prior to the addition of [^{32}P]PP_i, which was used to start the reaction. Exchange rates were normalized by attributing a value of 1 to the rate observed in the absence of cadaverine. The continuous curves were drawn according to the Monod−Wyman−Changeux model described in the text, with the parameters given in Table 2.

Table 2: Allosteric Constants for Lysine and Cadaverine of the ATP−PP_i Exchange Reaction Catalyzed by the T208M or T208A LysRS[a]

			K_R or K_{IR} (μM)	
LysRS	L_o	k_R (s^{-1})	lysine	cadaverine
T208M	105 ± 14	28 ± 2	89 ± 8	307 ± 45
T208A	63 ± 9	23 ± 2	61 ± 6	295 ± 36

[a] Initial rates of ATP−PP_i exchange were measured at 28 °C in a reaction mixture containing 20 mM Tris-HCl buffer (pH 7.8), 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 7 mM $MgCl_2$, 2 mM ATP, 2 mM [^{32}P]PP_i, and various concentrations of lysine and cadaverine. The enzyme was always preincubated for 5 min at 28 °C in the presence of all components of the reaction except [^{32}P]PP_i. The data were fitted according to the Monod−Wyman−Changeux model described in the text. In this model, L_o is the ratio between the concentrations of the T- and the R-forms in the absence of lysine (L_o = [T]/[R]); K_R and K_{IR} characterize the dissociation of lysine and cadaverine from the R-form, respectively; and k_R is the catalytic rate constant associated with the R-form.

complex (Segel, 1975) or on the concept of mnemonical enzymes (Ricard *et al.*, 1974) could also account for sigmoidal responses and activation by a competitive inhibitor. However, these mechanisms are not relevant here because the various LysRS:substrate complexes are in equilibrium when the steady state rate of the exchange reaction is reached.

The continous lines in Figures 2 and 3 show that the MWC model accounts for both the sigmoidal dependence of the reaction rate on the lysine concentration and the effect of cadaverine. The apparent dissociation constant of lysine from the R-form (K_R = 89 μM) is greater than that from the wild-type enzyme (1 μM) (Brevet *et al.*, 1995) by nearly 2 orders of magnitude (Table 2). The catalytic rate associated with the R-form (k_R = 28 s^{-1}) is equal to the k_{cat} measured above, *i.e.*, one-half the catalytic rate of the wild-type enzyme. The equilibrium constant between the T- and R-forms is 105, corresponding to a free energy difference of 2.8 kcal/mol. The dissociation constant of cadaverine from the R-form (K_{IR} = 307 μM) is greater by less than 1 order of magnitude than the K_I determined with the wild-type enzyme (50 μM) (Brevet *et al.*, 1995). This suggests that cadaverine binding is less affected than lysine binding in the mutant enzyme.

Aminoacylation of $tRNA^{Lys}$ by T208M LysRS. When aminoacylation of purified $tRNA^{Lys}$ by T208M LysRS was studied, several features of the reaction appeared similar to those of the ATP−PP_i exchange reaction. The amount of labeled lysyl-$tRNA^{Lys}$ did not increase linearly with time when the reaction was initiated by the simultaneous addition of 2 mM ATP, 2 mM lysine, and 5 μM $tRNA^{Lys}$. Again, the lag was suppressed when the enzyme was preincubated for 5 min with both ATP and lysine, before the initiation of the reaction by the addition of $tRNA^{Lys}$ (data not shown). In the presence of 2 mM ATP and 5 μM $tRNA^{Lys}$, the steady-state rate increased in a sigmoidal manner as a function of lysine concentration in the assay, in agreement with a cooperative binding of the amino acid to the enzyme. However, radiolabeled lysine concentrations higher than 0.5 mM could not be used in the tRNA aminoacylation assay. Therefore, saturating lysine concentrations could not be reached, and the catalytic parameters of the reaction could not be determined.

Dimerization of T208M LysRS. Since Thr 208 is likely to lie at the subunit interface, one way to explain the cooperative binding of lysine to T208M LysRS is to suppose that the mutant dimeric enzyme partly dissociates into subunits, and that the monomeric and dimeric forms of the enzyme exhibit different affinities for the amino acid. However, such a hypothesis seems unlikely, because (i) whatever the assay conditions used, the rate of the ATP−PP_i exchange reaction catalyzed by T208M LysRS increased linearly with the enzyme concentration, and (ii) analysis of the mutant LysRS by polyacrylamide gel electrophoresis under non-denaturating conditions or by molecular sieving on Sephacryl S300 never showed dissociation (data not shown). Notably, the latter experiment was performed at enzyme concentrations (40 nM) similar to those used in the exchange reaction.

Pre-Steady-State Kinetics. As mentioned above, the addition of the T208M mutant LysRS to a buffer containing 2 mM ATP, 2 mM lysine, and 2 mM labeled PP_i resulted in a lag of approximately 1 min before the steady-state rate of ATP−PP_i exchange was reached. This behavior, which was not observed with the wild-type enzyme, suggested the occurrence of a very slow step in the pathway from PP_i to ATP, or from ATP to PP_i. Because the steady-state rate of the exchange reaction (28 s^{-1}) is much faster than the time constant of the lag (0.02 s^{-1}), it may be concluded that the limiting reaction step no longer occurs after the lag period. Such a behavior of the mutant enzyme may *a priori* arise from one of the following mechanisms: (i) A slow time-limiting binding of one (or several) substrate(s) to the enzyme at the first round of ATP−PP_i exchange. However, a slow binding of ATP or PP_i can readily be excluded because it would not be compatible with the further observed rapid steady-state rate of ATP−PP_i exchange. A slow binding of ATP or PP_i to one subunit, followed by rapid binding to the other subunit, can also be excluded since such a behavior would lead to cooperative bindings of ATP or PP_i, which were not observed. (ii) A slow time-limiting rate of formation of the first molecule of lysyladenylate on one subunit followed by rapid synthesis of the second lysyl-adenylate on the second subunit. At the steady state, the

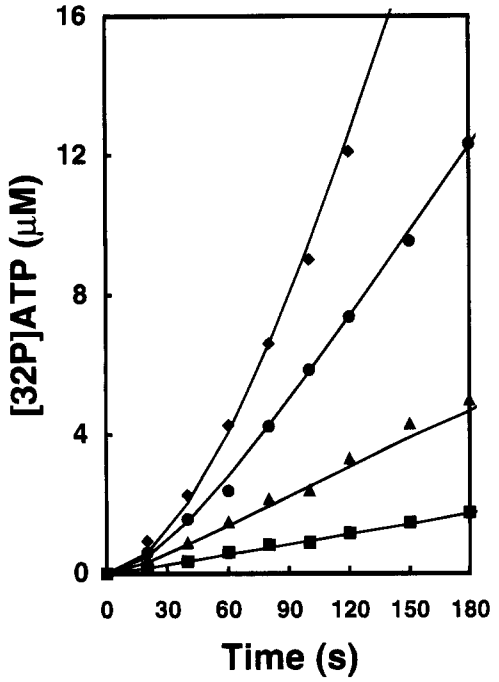


FIGURE 4: Time course of the [^{32}P]PP_i−ATP exchange reaction catalyzed by the T208M LysRS at various concentrations of lysine. The reaction mixture, buffered with 20 mM Tris-HCl (pH 7.8), contained 2 mM ATP, 2 mM [^{32}P]PP_i, 7 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, and either 0.1 (■), 0.2 (▲), 0.4 (●), or 0.8 mM lysine (◆). The reaction was started by the addition of 0.2 μM T208M LysRS. The continuous curves describing the number of [^{32}P]ATP molecules formed as a function of time were drawn according to the Monod−Wyman−Changeux model described in the text, with the parameters given in Table 2 and a k_{L_o} value of 0.007 s^{-1}.

enzyme would no longer be freed of lysyladenylate on one or the other protomer, thus always supporting a rapid ATP−PP_i exchange. (iii) A slow time-limiting conformational change of the enzyme population during the pre-steady state. In the context of the above mentioned MWC model, such a mechanism would correspond to a slow transition between the two enzyme conformers.

To distinguish between these various possibilities, the mutant enzyme was preincubated with various combinations of ligands prior to the initiation of the reaction 5 min later. The lag disappeared if the enzyme was preincubated with 2 mM ATP and 2 mM cadaverine, prior to the initiation of the exchange by the addition of 0.3 mM lysine and 2 mM PP_i (data not shown). This observation excluded that the lag may result from either a slow binding of lysine or a slow formation of the first lysyladenylate. Consequently, hypotheses (i) and (ii) were ruled out. In turn, this behavior supports the idea that the time-limiting step is caused by a slow transition between two conformers of the mutant enzyme (hypothesis (iii)).

To confirm this hypothesis, we attempted to fit the pre-steady-state kinetics using the above determined constants (Table 2) and a slow kinetic rate (k_{L_o}) for the transition from the T- to the R-form. A good agreement between experimental and theorical values could be obtained with k_{L_o} values nearby 0.007 s^{-1}, corresponding to an activation free energy of approximately 20 kcal/mol. Moreover, the model predicted that the lag must be reduced when lysine concentration is decreased. This could be experimentally verified (Figure 4). The lag became unmeasurable at 0.1 mM lysine.

Finally, we attempted to fit our data by supposing that the T-form was able to bind lysine and to catalyze the ATP−PP_i exchange reaction. With the introduction of these additional parameters, a satisfactory fit of both the steady-state and the pre-steady-state data required that the dissociation constant of lysine from the T-form be fixed high (K_T > 3 mM) and that the value of the catalytic efficiency of the T-form (k_T/K_T, where k_T designates the catalytic constant of the T-form) be taken to be at least 1000-fold lower than that of the R-form. These calculations *a posteriori* justified the initial assumption that lysine binding to the T-form could be neglected in the analysis of the steady-state kinetics.

Kinetic Studies of the T208A Mutant Enzyme. To determine whether the properties of the T208M mutant originated from the bulky side chain of the methionine introduced as residue 208 rather than from the loss of the threonine residue, a mutant LysRS where Thr 208 was changed into an alanine, was constructed by site-directed mutagenesis.

The properties of this T208A mutant were similar to those of T208M LysRS. The rate of the ATP−PP_i exchange reaction was time-dependent, and the steady-state rate depended on lysine concentration in a sigmoidal manner (Figure 2). The k_{cat} value of T208A LysRS, as well as the K_m values for ATP and PP_i, differed by less than a factor of 2.5 from the corresponding value measured with T208M LysRS (Table 1). The Hill coefficients characterizing the dependence of the reaction rate on lysine concentration were similar for T208A LysRS (1.55) and T208M LysRS (1.65). When the kinetics were fitted according to the allosteric model described above, the apparent dissociation constants of lysine and cadaverine (K_R = 61 μM, and K_{IR} = 295 μM) appeared roughly equivalent to the values found for T208M LysRS. In contrast, the equilibrium constant of T208A LysRS (L_o = 64) was affected by a factor of nearly 2 when compared to T208M LysRS. This L_o value corresponds to a free energy difference of 2.46 kcal/mol.

The slow kinetic rate k_{L_o} for the transition from the T- to the R-form associated with the T208A mutant could be derived from the pre-steady-state data. The value obtained for this mutant (k_{L_o}= 0.009 s^{-1}) was nearly the same as that obtained for the T208M mutant.

DISCUSSION

Until this work, the role of dimerization of class II aaRS subunits has only been probed in the case of the yeast aspartic system (Eriani *et al.*, 1993), closely related to the lysine system. The mutagenesis of the invariant proline residue of motif 1 showed the structural importance of the AspRS dimer interface. Thus, the P273G mutant AspRS exhibited ATP−PP_i exchange and tRNA aminoacylation k_{cat} values 6 and 3 times lower than those of the native enzyme, respectively. However, the mutated subunit recovered wild-type enzymatic properties when associated with a native subunit, thereby establishing the functional interdependence between the two AspRS subunits. Structural data suggest that the motif 1 residues of AspRS are closely related to the active sites of both subunits by a network of hydrogen bonds and salt bridges. The residues of the short β-strand, in which lies the invariant proline, are close to motif 2 residues, especially arginine 325 and glutamine 303. The latter residue is involved in the binding of the aspartate substrate, whereas Arg 325 would stabilize the α-phosphate of ATP (Cavarelli

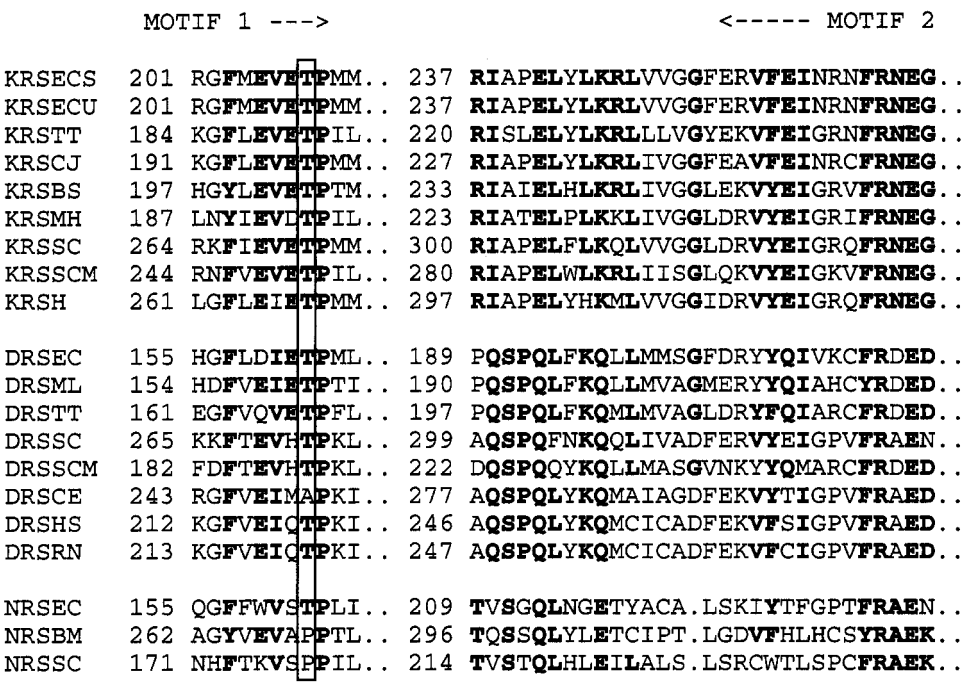


FIGURE 5: Partial alignment of lysyl-, aspartyl- and asparaginyl-tRNA synthetase sequences. Shown are the sequences of motif 1 and of the region located upstream of motif 2 for *E. coli lysS* gene product (KRSECS) and *E. coli lysU* gene product (KRSECU); LysRS from *Thermus thermophilus* (KRSTT), *Campylobacter jejuni* (KRSCJ), *Bacillus subtilis* (KRSBS), and *Mycoplasma hominis* (KRSMH); LysRS from *Saccharomyces cerevisiae* cytoplasm (KRSSC) or mitochondria (KRSSCM); LysRS from Chinese hamster (KRSH); AspRS from *E. coli* (DRSEC), *Mycobacterium leprae* (DRSML), and *Thermus thermophilus* (DRSTT); AspRS from *S. cerevisiae* cytoplasm (DRSSC) or mitochondria (DRSSCM); AspRS from *Caenorhabditis elegans* (DRSCE), man (DRSHS), and rat (DRSRN); and AsnRS from *E. coli* (NRSEC), *Brugia malayi* (NRSBM), and *S. cerevisiae* cytoplasm (NRSSC). The *B. subtilis* LysRS and *C. elegans* AspRS genes were identified through homologies with other LysRSs and AspRSs, in the course of the systematic sequencing of the corresponding genomes. In the case of AsnRS from *B. malayi*, the sequence used is that of an antigenic protein showing high homologies with *E. coli* and *S. cerevisiae* AsnRSs. As previously discussed (Cusack *et al.*, 1991), a canonical motif 2 can be recognized in this sequence if one assumes a frameshift sequencing error. Numbers indicate the amino acid positions relative to the N-termini of the corresponding proteins. Dots represent gaps inserted to optimize the alignments. Conserved residues are shown in boldface characters, and the mutated threonine of *E. coli* LysRS is boxed.

et al., 1994). Data from the *E. coli* SerRS structure also suggest that the short β-strand of motif 1 stabilizes the active site conformation by forming main-chain hydrogen bonds with a β-strand of motif 2 (Cusack *et al.*, 1991).

In this study, the cloning of a mutant LysRS affected in lysine binding reveals the importance of a conserved Thr residue of motif 1 (Figure 5). Substitution of this threonine with a methionine residue triggers cooperativity for lysine binding. Moreover, the time course of the ATP–PP_i exchange reaction catalyzed by the mutant enzyme displays a significant lag when addition of the enzyme is used to start the reaction. The behavior of the T208M LysRS does not result from the greater bulkiness of the methionine compared to threonine since the T208A LysRS displays the same characteristics. The cooperative behavior and the pre-steady-state time lag of these mutant enzymes can be satisfactorily accounted for by a model in which the lysine binding sites on both subunits have been disturbed. Upon saturation by the amino acid, the enzyme would undergo a concerted conformational change of both subunits, as described in the MWC model. According to this model, the mutant enzymes obey a T $\rightarrow$ R transition. The associated activation free energy (20 kcal/mol) indicates a rather important structural rearrangement, although the differences in stabilities between the R- and T-states is less than 3 kcal/mol, i.e., roughly the free energy value of a hydrogen bond.

The recently solved 3-D structure of *E. coli* LysRS (the *lysU* gene product) shows a Thr208 residue located near the dimer interface, thus far away from the bound lysine substrate (about 12 Å) (Onesti *et al.*, 1995). However, the hydroxyl group of Thr 208 points toward the guanidino group of Arg 237. This Arg residue is located just upstream of an α-helix interacting with the lysine substrate through the side chains of Glu 241 and Lys 245. All of these residues are conserved in the sequence of the product of *lysS*. Moreover, the amino acid sequence of this region is particularly well conserved among the homologous aaRSs corresponding to either lysine, aspartate, or asparagine (Figure 5). Structural data and site-directed mutagenesis experiments in the aspartic system strongly suggest the involvement of this region in amino acid binding (Cavarelli *et al.*, 1994; Poterszman *et al.*, 1994). This region, which immediately precedes motif 2, was in fact also shown to participate to the binding of several seryladenylate analogs in *T. thermophilus* SerRS (Belrhali *et al.*, 1994).

The possibility to convert a Michaelian enzyme into a cooperative one has recently been assessed by site-directed mutagenesis. Upon the substitution of a limited number of active site residues, *E. coli* ornithine transcarbamoylase (Kuo *et al.*, 1989) and *Bacillus subtilis* aspartate transcarbamoylase (Stebbins & Kantrowitz, 1992) could be given a cooperative behavior. However, these enzymes belong to a family already displaying several well-described cooperative members such as the *E. coli* aspartate transcarbamoylase (Hervé, 1989). In the case of *Bacillus stearothermophilus* TyrRS, positive cooperativity toward ATP binding could be induced by mutating a lysine residue in the active site (First & Fersht,

1993). This positive cooperativity would occur in the wild-type enzyme, but would normally not be visible because binding of the first ATP molecule would be followed by formation of tyrosyladenylate before an ATP molecule binds to the other site. The induction of cooperativity through engineering of residues of the subunits interface, far from the active site, was first reported in the case of *E. coli* glutathione reductase (Scrutton *et al.*, 1992). This enzyme becomes highly cooperative (Hill coefficient of 1.76) for glutathione binding upon changing its Gly418 residue into a bulky tryptophan. Similarly, substitution of a cysteine residue in the C-terminal domain of *E. coli* AlaRS introduced positive cooperativity toward tRNA binding (Wu *et al.*, 1994). In the latter two cases, the effects of the mutations were attributed to a disruption of the atom packing at the dimer interface, able to promote a change of the kinetic mechanism. In the present study, a similar consequence follows the substitution of Thr 208 by either methionine or alanine.

Dimeric class II synthetases usually follow Michaelian kinetics. Therefore, the functional advantage of the dimeric association of these synthetases is not readilly apparent. In the case of *T. thermophilus* SerRS (Biou *et al.*, 1994), the binding of one tRNA molecule requires the two subunits. This is not the case, however, in the aspartic system (Ruff *et al.*, 1991). One possibility might be that the dimeric character of the class II synthetases contributes to the stabilization of the subunits in a productive conformation. The properties of the mutant LysRSs studied here support this idea.

The OEL134 strain behaves as a lysine bradytroph. Likely, when the strain is grown in minimal medium, the cellular lysine concentration is not high enough to force the mutated LysRS into the R-conformation. Consequently, the limiting activity of the mutant enzyme would maintain the lysylation of tRNA at a low level (Boy *et al.*, 1978) and the growth rate would be impaired. Upon addition of 10 mM lysine in the culture medium, a normal growth rate is restored. This effect may be attributed to the binding of lysine to the T-species, capable of shifting LysRS conformation in the productive direction. Therefore, the single-point T208M mutation could be enough to render protein biosynthesis strongly sensitive to the presence or absence of external lysine.

Clearly, enzyme cooperativity having strong regulatory consequences may result from minor molecular switches, even away from the active site. This tends to indicate that the evolution of a metabolic pathway can be sustained by rather small mutagenic events, susceptible to occur at a high frequency in the cell progeny.

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