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# Modification of aminoacyl-tRNA synthetases with pyridoxal-5'-phosphate. Identification of the labeled amino acid residues

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**Summary** — The isotopic [$^{32}$P]PPi-ATP exchange activity of isoleucyl-, valyl-, histidyl-, tyrosyl- and methionyl-tRNA synthetases from *Escherichia coli* are lost upon incubation in the presence of pyridoxal-5'-phosphate (PLP). When the residual activity of either isoleucyl-, valyl- or methionyl-tRNA synthetase (monomeric truncated form) was plotted as a function of the number of PLP molecules incorporated per enzyme molecule, the plots obtained appeared biphasic. Below 50% inactivation of these enzymes, PLP incorporation varied linearly with the isotopic exchange measurements, and extrapolation of the first half of the plot indicated a stoichiometry of 1.10 ± 0.05 mol of PLP incorporated per mol of 100% inactivated synthetase. Beyond 50% inactivation, the graph deviated from its initial slope, and up to 4–5 mol of PLP were incorporated per mol of synthetase at the highest used PLP concentrations. In the cases of homodimeric histidyl- and tyrosyl-tRNA synthetases, extrapolation of the graph at 100% inactivation indicated 2.8 ± 0.1 and 2.4 ± 0.1 mol of PLP incorporated per mol of enzyme, respectively. PLP-labeled peptides were obtained through trypsin digestion and RPLC purification, prior to Edman degradation analysis. PLP-labeled residues were identified as lysines 132, 332, 335 and 402 of monomeric methionyl-tRNA synthetase, lysines 332, 335, 402, 465, 596 and 640 of native dimeric methionyl-tRNA synthetase, lysines 22, 117, 601, 604 and 645 of isoleucyl-tRNA synthetase, lysines 554, 557, 559, 593 and 909 of valyl-tRNA synthetase, lysines 2, 118, 369 and 370 of histidyl-tRNA synthetase and lysine 237 of tyrosyl-tRNA synthetase. In addition, the amino terminal residue of the polypeptide chain(s) of either isoleucyl-, valyl-, histidyl- or methionyl-tRNA synthetases was found labeled. Among these residues, lysines 332, 335 and 402 of monomeric methionyl-tRNA synthetase as well as lysines 332, 335, 402 and 596 of dimeric methionyl-tRNA synthetase, lysines 601, 604 and 645 of isoleucyl-tRNA synthetase, lysines 554, 557 and 559 of valyl-tRNA synthetase, lysines 2, 369 and 370 of histidyl-tRNA synthetase, and lysine 237 of tyrosyl-tRNA synthetase were labeled in the presence of PLP concentrations smaller than or equal to 1 mM, and are shown to be critical for the activity of the enzymes. It is concluded that these residues participate to the binding sites of the phosphates of ATP on the studied synthetases.

**aminoacyl-tRNA synthetases / pyridoxal 5'-phosphate / labeled lysyl residues**

## Introduction

Aminoacyl-tRNA synthetases catalyze each the activation, at the expense of ATP, of a specific amino acid and the subsequent transfer of this amino acid to the 3' terminus of cognate isoacceptor tRNAs [1]. Because of the anionic nature of ATP and tRNA, positively charged amino acid residues are expected to be present within the active center of these enzymes. Indeed, lysine residues have been evidenced in the tRNA- and ATP-binding sites of several aminoacyl-tRNA synthetases [2–12]. In particular, affinity labeling of *E coli* methionyl-tRNA synthetase (MetRS) with periodate-oxidized $tRNA^{Met}$ identified Lys-61, Lys-142, Lys-147, Lys-149, Lys-335 and Arg-435 as parts of the binding site of the CCA end of $tRNA^{Met}$ [5, 8]. More recently, lysine residues involved in the binding of the γ-phosphate of ATP to *E coli* MetRS or valyl-tRNA synthetase (ValRS) have been mapped by using pyridoxal 5'-triphospho-5'-adenosine ($AP_3$-PL) as an affinity label [9]. $AP_3$-PL was found attached to either lysine 335, 402 or 528, in the primary structure of MetRS. In the case of ValRS, the $AP_3$-PL-labeled residues were lysines 557, 593 and 909.

Moreover, the aminoacyl-tRNA synthetases specific for isoleucine, leucine, methionine, valine, cysteine,

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*Abbreviations :* PLP, pyridoxal 5'-phosphate ; PTH, phenylthiohydantoin ; $NaBH_4$, sodium borohydride ; TFA, trifluoroacetic acid ; RPLC, reverse phase liquid chromatography. Aminoacyl-tRNA synthetases are abbreviated as a three-letter code of their specific amino acid followed by RS ; the one- and three-letter amino acid codes are those suggested by IUPAB-IUB Commission on Biochemical Nomenclature.

tryptophane, tyrosine, arginine, glutamine and glutamic acid display a lysine-rich sequence, the consensus of which is KMSKS [5, 6, 9, 13, 14]. In the cases of methionyl- and tyrosyl-tRNA synthetases, site-directed mutagenesis experiments have pinpointed the involvement of the KMSKS sequence in the stabilization of the transition state during the synthesis of aminoacyl-adenylate, *via* the interaction of a lysine with the pyrophosphate moiety of the ATP molecule [15, 16]. The occurrence of both this sequence and the HIGH signature sequence [17] designated a particular class of aminoacyl-tRNA synthetases called class 1 [18, 19].

Another set of aminoacyl-tRNA synthetases called class 2, includes AlaRS, AsnRS, AspRS, GlyRS, HisRS, LysRS, PheRS, ProRS, SerRS and ThrRS. They all lack the HIGH and KMSKS consensus sequences and, instead, show three conserved sequence motifs [18–22]. In the latter class of synthetases, residues involved in the binding of ATP or of tRNA have been searched for by affinity labeling studies only in the cases of PheRS [7, 12] and AlaRS [11].

Another powerful reagent widely used to identify lysine residues at the nucleotide binding site on enzymes, especially at the sub-sites of the phosphate groups, is pyridoxal-5'-phosphate (PLP) [23–27]. Labeling with this reagent results from Schiff base formation between its aldehyde function and the ε-amino group of a lysine residue in the protein. In many cases, labeling with PLP was shown to fulfill the general criteria of an affinity labeling reaction [23, 25, 26]. The labeling and inactivation of IleRS, MetRS and ValRS with PLP have already been described [28]. However, the lysine residues of the labeled synthetases were not further identified.

In the present study, the reaction of PLP with various aminoacyl-tRNA synthetases belonging to either of the two above classes is systematically followed. In most studied cases, the synthetases loose their activity while PLP molecules become covalently attached. The analysis of the labeled lysine residues as a function of the degree of enzyme inactivation enables us to identify the residues critical for activity, and conclusions are drawn for a further use of PLP as a general affinity labeling reagent in the characterization of aminoacyl-tRNA synthetases.

## Materials and methods

*Enzymes from E coli*

Homogeneous native methionyl-tRNA synthetase ($MetRS_N$) was purified from the overproducing strain PAL 1803.5 carrying recombinant plasmid pX1 [29]. The fully active monomeric truncated methionyl-tRNA synthetase (M547) was obtained as described [30]. Native valyl-tRNA synthetase (ValRS) was overexpressed and prepared homogeneously according to [31]. Isoleucyl-, histidyl- and tyrosyl-tRNA synthetases from *E coli* strain EM20031 were purified as described [32]. The molar concentration of each aminoacyl-tRNA synthetase was calculated from its absorbancy at 280 nm and from the following values of molecular ratio and of optical extinction coefficient deduced from amino acid composition: 64 K and 1.42 units $mg^{-1}$ $cm^2$ for M547, 152 K and 1.18 units $mg^{-1}$ $cm^2$ for $MetRS_N$, 105 K and 1.7 units $mg^{-1}$ $cm^2$ for IleRS, 101 K and 1.53 units $mg^{-1}$ $cm^2$ for ValRS, 95 K and 0.98 units $mg^{-1}$ $cm^2$ for TyrRS and 94 K and 1.09 units $mg^{-1}$ $cm^2$ for HisRS.

*Chemicals*

Pyridoxal-5'-phosphate (PLP) and TPCK-treated trypsin were purchased from Sigma.

*Labeling with pyridoxal-5'-phosphate (PLP)*

Aminoacyl-tRNA synthetases (13 μM) in 50 μl were incubated with PLP at 25°C in 50 mM phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol. Concentrations of PLP in the assay ranged from 0.016 to 16 mM. After 20 min (unless otherwise specified), a 40 μl aliquot of each reaction mixture was withdrawn and mixed with 40 μl of 0.1 M sodium borohydride ($NaBH_4$) freshly prepared in 10 mM NaOH. The reduction by $NaBH_4$ was allowed to proceed for 20 min. 5 μl of each $NaBH_4$-quenched sample were diluted with 1 ml Tris-HCl buffer (pH 7.6) containing 10 mM 2-mercaptoethanol and 200 μg/ml bovine serum albumin. Then the diluted enzyme solutions were assayed for amino acid-dependent isotopic [$^{32}$P]PPi-ATP exchange activity [33]. In parallel, 50 μl of the $NaBH_4$-quenched samples were applied to a TSK 4000 PW column (300 x 7.5 mm) equilibrated in 0.2 M $NH_4HCO_3$ (pH 8). The flow rate was 0.5 ml/min. Elution was monitored by the absorbance at 325 nm and by fluorescence (excitation at 280 nm and emission recorded at 330 nm). The amount of PLP bound to the enzyme was deduced from the light absorbancy at 325 nm by using a molar absorption coefficient at pH 8 of 7800 $M^{-1}$ $cm^{-1}$ [34].

*Substrate protection of MetRS from inactivation by PLP*

Substrate protection of M547 from inactivation was carried out at 25°C in 50 mM phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol. The enzyme (6 M in 50 μl) was preincubated for 5 min with 5 mM of either studied ligand, or couple of ligands, before adding 1.5 mM PLP.

*Preparation of phosphopyridoxylated synthetases and peptides.*

Aminoacyl-tRNA synthetases (13 μM) in 100 μl were incubated in the presence of 4 mM PLP at 25°C in 50 mM phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol. After stopping the incubation by the addition of 5 μl of 1 M $NaBH_4$, an aliquot (2 μl) was withdrawn, diluted and assayed for the amino acid-dependent isotopic [$^{32}$P]PPi-ATP exchange activity. In order to remove unreacted reduced PLP, the PLP-labeled synthetase was dialyzed overnight at 4°C against 1 l of 0.1 M $NH_4HCO_3$ (pH 8). PLP-labeling of aminoacyl-tRNA synthetases for peptide preparation was also performed using variable PLP concentrations. The phosphopyridoxylated proteins were digested overnight at 37°C with TPCK-treated trypsin, at a protease to substrate ratio of 1/50 (w/w).

*Purification of phosphopyridoxylated peptides.*

The tryptic digest obtained as above was applied to a Merck Superspher $C_{18}$ (reverse-phase) column (250 x 4 mm) equilibrated at 42°C with 0.1% trifluoroacetic acid (TFA) in water (pH 2). The peptides were eluted with two consecutive linear gradients of acetonitrile in water (0–32% during 60 min and 32–48% during 5 min). The flow rate was 0.9 ml/min and fractions of 0.9 ml were collected. Equivalent fractions from several runs were pooled for further purification, prior to Edman degradation analysis. The pools were then injected onto the same column equilibrated at 42°C with 0.1 % TFA. In this case, the separation step was performed using a flow rate of 1.2 ml/min and a gradient of acetonitrile with a slope of 0.16 % $min^{-1}$ during the adequate time interval. Peaks were detected by recording the absorbances at 215 nm and 325 nm, and the fluorescence at 395 nm (excitation at 335 nm). The labeled peptides concentrations were deduced from the absorbancy of solutions at 325 nm, using a molar absorption coefficient of 2000 $M^{-1}$ $cm^{-1}$ at pH 2 [34].

*Peptide sequencing*

Automated Edman degradation was carried out on a gas-phase sequencer (Applied Biosystems, model 470 A). Aliquots (60 µl) of 100–500 pmol of peptide in 50% acetonitrile were loaded on a glass fiber filter previously treated with 2.5 mg of Biobrene (Applied Biosystems). PTH-amino acids were identified as already described [35].

## Results

### *Aminoacyl-tRNA synthetases loose activity in the presence of PLP*

IleRS, ValRS, HisRS, TyrRS, M547 or $MetRS_N$ were each incubated at 25°C (pH 7.0) in the presence of various concentrations of PLP. After reduction, protein samples were assayed for isotopic [$^{32}$P]PPi-ATP exchange activity in the presence of the cognate amino acid. The activities of IleRS, ValRS, HisRS, TyrRS, M547 and $MetRS_N$ were all sensitive to the presence of PLP, the degree of their inactivation depending on both the PLP concentration and the time of incubation. A typical experiment, performed with M547 (monomeric truncated MetRS), is shown in figure 1A. While the activity remained stable over 40 min in the absence of PLP, it decreased rapidly within 10 min in the presence of the reagent, reaching a stable plateau value depending on the concentration of PLP in the incubation mixture. This plateau value reflects the Schiff base equilibrium at the pH of the experiment. The further addition of $NaBH_4$ reduces both the unreacted PLP and the imine bond formed between PLP and the enzyme. After 20 min in the presence of 4 mM PLP, $MetRS_N$, M547, IleRS, ValRS, HisRS and TyrRS (13 µM each) retained 9%, 10%, 8%, 7%, 69% and 39% of their initial activity, respectively.

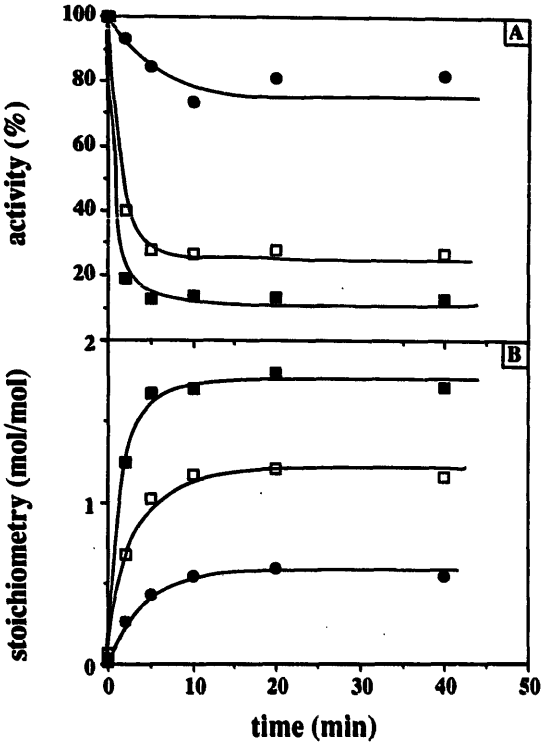


**Fig 1.** Inactivation of M547 by pyridoxal-5'-phosphate. **A.** The reaction mixture consisted of 13 µM of M547, 50 mM phosphate buffer (pH 7.0), 10 mM 2-mercaptoethanol and pyridoxal-5'-phosphate at 0.25 (●), 1.0 (❑) or 4 mM (■). At various times during a 40-min incubation at 25°C, aliquots were withdrawn, reduced by $NaBH_4$, diluted and assayed for isotopic [$^{32}$P]$PP_i$-ATP exchange activity, as described in *Materials and methods*. During this time interval, the activity of the enzyme incubated in the absence of PLP remained stable. The residual enzyme activity, expressed as a percent of the activity in the absence of PLP, was plotted as a function of the incubation time. **B.** In parallel with enzymatic activity measurements, the number of mol of pyridoxal-5'-phosphate incorporated per mol of M547 was deduced from light absorbancy measurement at 325 nm, by using a molar absorption coefficient at pH 8 of 7800 $m^{-1}$ $cm^{-1}$ [34]. The stoichiometries (mol/mol) were plotted as a function of the incubation time.

### *Aminoacyl-tRNA synthetases covalently bind PLP*

For all studied synthetases, the number of incorporated PLP molecules in the presence of different free PLP concentrations (0.016–16 mM) were measured at equilibrium of the Schiff base (reaction time of 20 min). A typical experiment with M547 is shown in figure 1B. In figure 2, the residual activity of either IleRS, ValRS, M547, HisRS or TyrRS is plotted as a function of the number of PLP molecules incorporated per molecule of enzyme.

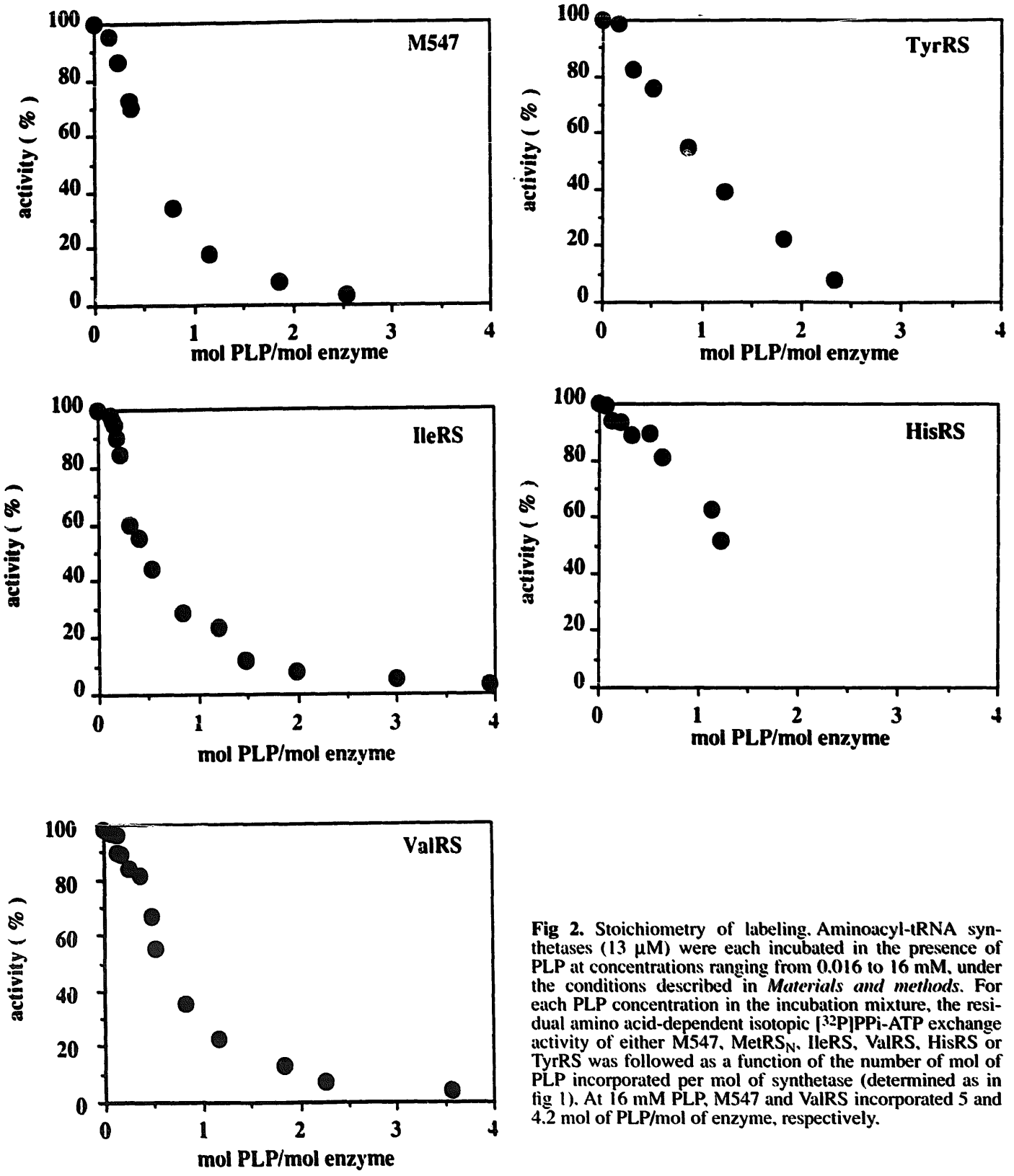


**Fig 2.** Stoichiometry of labeling. Aminoacyl-tRNA synthetases (13 μM) were each incubated in the presence of PLP at concentrations ranging from 0.016 to 16 mM, under the conditions described in *Materials and methods*. For each PLP concentration in the incubation mixture, the residual amino acid-dependent isotopic [$^{32}$P]PPi-ATP exchange activity of either M547, $MetRS_N$, IleRS, ValRS, HisRS or TyrRS was followed as a function of the number of mol of PLP incorporated per mol of synthetase (determined as in fig 1). At 16 mM PLP, M547 and ValRS incorporated 5 and 4.2 mol of PLP/mol of enzyme, respectively.

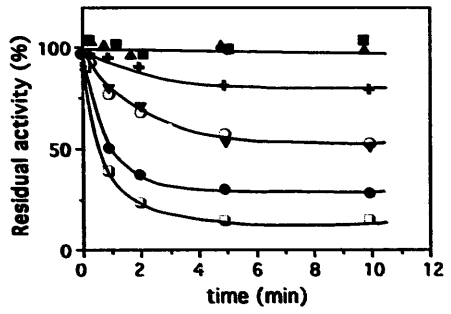


**Fig 3.** Effects of substrates on the inactivation of M547 by PLP. The enzyme (6 μM) in 50 mM phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol, was preincubated during 5 min in the absence (❑) or the presence of 5 mM AMP (●), 5 mM ATP (✚), 5 mM MgATP (▼), 5 mM L-methioninol (○), 5 mM L-methioninol + 5 mM AMP (■), or 5 mM L-methioninol + 5 mM MgATP (■). Afterward the inactivation was initiated by the addition of 1.5 mM PLP and, followed at various times, during a 10 min-incubation at 25°C, by the withdrawing of aliquots and the measurement of their activity after reduction by $NaBH_4$, as described in *Materials and methods*. Control experiments (▲) without PLP are also shown.

In the cases of IleRS, ValRS and MetRS (monomeric M547 species) the plots of figure 2 appeared biphasic. Below 50% inactivation of each of these enzymes, PLP incorporation varied linearly with the isotopic exchange measurements. A stoichiometry of 1.10 ± 0.05 mol of PLP incorporated per mol of 100% inactivated IleRS, ValRS or M547 can be deduced through extrapolation of the first half of the plots. This result is consistent with the occurrence of one active site in either IleRS, ValRS or M547 [36–40], and suggests at the same time that the amino acid residues labeled at low PLP concentrations interfere with enzyme activity. Beyond 50% inactivation of either IleRS, ValRS or M547, the graph deviates from its initial slope, indicating that up to 4–5 mol of PLP could be incorporated per mol of each studied synthetase at the highest used PLP concentrations.

The behaviour of HisRS and TyrRS was slightly different since the incorporation of PLP varied linearly with the residual activity whatever the concentration of PLP in the assay. Extrapolation of the graphs at 100% inactivation indicated 2.8 ± 0.1 and 2.4 ± 0.1 mol of PLP incorporated per mol of dimeric HisRS or TyrRS, respectively (fig 2).

After a prolonged incubation (90 min) in the presence of 16 mM PLP, HisRS still retained 50% of its initial activity, while about 1 mol of reagent was incorporated per mol of dimer (fig 2). Increasing the pH of the incubation mixture (up to pH 9.0) did not result in increased enzyme inactivation or PLP incorporation (results not shown). However, the use of $NaBH_3CN$, a mild reducing agent for the *in situ* reduction of the Schiff base, significantly increased the extent of enzyme inactivation and the degree of PLP incorporation (67% and 1.3 mol of PLP, respectively, in 50 mM phosphate buffer, pH 7.0) (results not shown). This suggests that partial HisRS inactivation and labeling is due to the equilibrium of the Schiff base, as already discussed [4]. Indeed, when added to the incubation mixture, $NaBH_3CN$ is able to specifically and continuously displace the Schiff base equilibrium, while leaving intact the reacting aldehyde group of PLP.

### *Substrate protection of MetRS from inactivation by PLP*

The effects of substrates on the inactivation of M547 by PLP were examined. As shown in figure 3, incubation of the enzyme (6 μM in a 50 μl-incubation mixture) with 1 mM PLP in the presence of either AMP, MgATP or ATP at 5 mM each resulted in the loss of enzyme activity by 70%, 47% and 20%, respectively, instead of 85% in the control experiment without protecting substrate. L-methioninol, the analog of methionine in which the carboxylate is replaced by a $CH_2OH$ group, afforded some protection. In fact, this apparent protection was the result of the reaction of PLP with the α-amino group of L-methioninol (5 mM), as demonstrated by HPLC analysis of PLP incubated with this methionine analog and reduced with $NaBH_4$ (not shown). However, when combined with MgATP (5 mM) or AMP (5 mM), L-methioninol (5 mM) completely protected the enzyme from inactivation (fig 3). As already discussed [9], protection by L-methioninol in the presence of MgATP or AMP can be accounted for by the synergistic coupling of the binding of this amino alcohol with the binding of either nucleotide within the adenylation site of M547 [41–43], and suggests at the same time that inactivation by PLP proceeds through prior binding of the reagent to the active site of the enzyme.

### *PLP-labeled peptides of monomeric MetRS (M547)*

PLP-labeled peptides were obtained through trypsin digestion and RPLC purification. Figure 4 shows a typical separation profile of the PLP-labeled peptides of 90% inactivated monomeric truncated MetRS (13 μM M547, incubated 20 min in the presence of 4 mM PLP). Five main well-resolved peaks, numbered $m_1$–$m_5$ were recovered. The corresponding amino acid sequences were determined by Edman degradation. Systematically, the expected phenylthiohydantoin (PTH) derivative of PLP-labeled lysine was not visible on the chromatogram of PTH analysis. As already discussed by Sejlitz *et al* [27], this may be caused by the strong adsorption of the phosphate group of PLP to the polybrene-treated glass-fibre

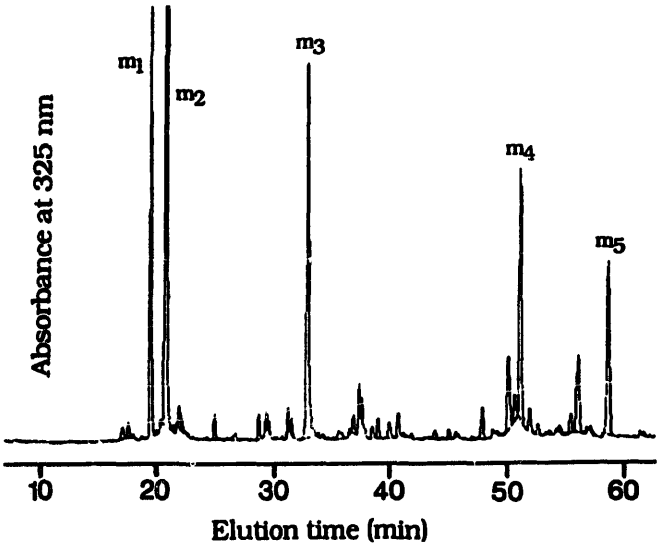


**Fig 4.** High-performance liquid chromatography of the PLP-labeled peptides of M547. The tryptic digest of 83 µg of M547 labeled for 20 min in the presence of 4 mM PLP, was subjected to high-performance chromatography on a Merck Superspher $C_{18}$ (reverse-phase) column, as described in *Materials and methods*. The flow rate was 0.9 ml/min. The peptides were eluted with two consecutive linear gradients of acetonitrile in 0.1% trifluoroacetic acid (0–32% during 60 min and 32–48% during 5 min). Absorbance at 325 nm was monitored.

filter. Peptides $m_1$, $m_3$, $m_4$ and $m_5$ corresponded respectively to positions 333–337 (MSKSR), 396–403 (NAGFINKR), 316–335 (KPSNLFVHGYVTVNGAKMSK) and 123–139 (TISQLYDPEKGMFLPDR) in the sequence of M547 [29], and the PLP-labeled amino acid residues of peptides $m_1$, $m_3$, $m_4$ and $m_5$ were identified as Lys-335, Lys-402, Lys-332 and Lys-132, respectively (table I).

During the automated Edman degradation of peptide $m_2$, no PTH- aminoacid was visible at cycle 1, while, at further cycles, the sequence TQVAK could be read, despite a low PTH signal and a considerable overlap from one cycle to the following. This suggested that peptide $m_2$ was blocked at its $NH_2$-terminus, and that the release of a small amount of PLP from the peptide by the strong acidic treatment in the sequencer has permitted the fixation of the Edman reagent on the $NH_2$-terminus and the subsequent degradation of the peptide. Accordingly, peptide $m_2$ corresponded to positions 1–5 (TQVAK), with the α-amino group of Thr-1 labeled with PLP. Amino acid analysis of peptide $m_2$ (not shown) confirmed these results.

*PLP-labeled peptides of native MetRS*

As expected, most of the PLP-labeled peptides of M547 were also found labeled in the native dimeric methionyl-tRNA synthetase (13 µM $MetRS_N$, 91% inactivated within 20 min in the presence of 4 mM PLP). These peptides, numbered $M_1$–$M_4$, corresponded respectively to positions 333–337 (MSKSR), 1–5 (TQVAK), 396–403 (NAGFINKR) and 322–335 (VHGYVTVNGAKMSK) in the sequence of the enzyme [29]. PLP-labeled residues of peptides $M_1$–$M_4$ were Lys-335, Thr-1 (labeled on its α-amino group), Lys-402 and Lys-332, respectively (table I). Noteworthy, Lys-132 was not found labeled.

In addition, three new PLP-labeled peptides (numbered $M_5$–$M_7$) were identified, two of which ($M_5$ and $M_7$) originated from the carboxyl-terminus extension of the protomer of the native MetRS, as compared with the truncated M547 enzyme. Peptides $M_5$–$M_7$ corresponded respectively to positions 640–642 (KMR), 462–469 (VVAKQEGR) and 582–599 (VALIENAEFVEGSDKLLR) in the sequence of MetRS [29], with the PLP covalently attached to Lys-640, Lys-465 and Lys-596, respectively (table I).

*PLP-labeled peptides of IleRS*

The PLP-labeled peptides of 92% inactivated isoleucyl-tRNA synthetase (13 µM IleRS, incubated 20 min in the presence of 4 mM PLP) were recovered from six peaks, numbered $I_1$–$I_6$. Peptides $I_1$–$I_6$ corresponded to positions 1–4 (SDYK), 601–604 (KMSK), 18–23 (GDLAKR), 115–121 (VEKEYGK), 602–618 (MSKSIGNTVSPQDVMNK) and 626–646 (LWVASTDYTGQMAVSDEILKR) in the primary structure of IleRS [44]. The PLP-labeled residues of peptides $I_1$–$I_6$ were Ser-1 (labeled on its α-amino group), Lys-601, Lys-22, Lys-117, Lys-604 and Lys-645, respectively (table I).

*PLP-labeled peptides of ValRS*

The PLP-labeled peptides of 93% inactivated valyl-tRNA synthetase (13 µM ValRS, incubated 20 min in the presence of 4 mM PLP) were recovered in six main peaks numbered $V_1$–$V_6$. Peptides $V_1$–$V_6$ corresponded respectively to positions 555–559 (MSKSK), 1–3 (MEK), 549–557 (DDEGQKMSK), 590–595 (LADKIR), 906–918 (IENKLANEGFVAR) and 558–580 (SKGNVIDPLDMVDGISLPELLEK) in the primary structure of ValRS [45, 46]. The PLP-labeled residues of peptides $V_1$–$V_6$ were Lys-557, Met-1 (labeled on its α-amino group), Lys-554, Lys-593, Lys-909 and Lys-559, respectively (table I).

**Table I.** Amino acid sequences of PLP-labeled peptides of M547, $MetRS_N$, IleRS, ValRS, HisRS and TyrRS[a].

| *Synthetase* | *amino acid sequence determined* | *Labeled residue* |
|---|---|---|
| | $^{1}$TQVAK$^{5}$ | N-term |
| | $^{123}$TISQLYDPEKGMFLPDR$^{139}$ | K132 |
| **M547** | $^{316}$KPSNLFVHGYVTVNGAKMSK$^{335}$ | K332 |
| | $^{333}$MSKSR$^{337}$ | K335 |
| | $^{396}$NAGFINKR$^{403}$ | K402 |
| | $^{1}$TQVAK$^{5}$ | N-term |
| | $^{322}$VHGYVTVNGAKMSK$^{335}$ | K332 |
| | $^{333}$MSKSR$^{337}$ | K335 |
| **MetRS** | $^{396}$NAGFINKR$^{403}$ | K402 |
| | $^{462}$VVAKQEGR$^{469}$ | K465 |
| | $^{582}$VALIENAEFVEGSDKLLR$^{599}$ | K596 |
| | $^{640}$KMR$^{642}$ | K640 |
| | $^{1}$SDYK$^{4}$ | N-term |
| | $^{18}$GDLAKR$^{23}$ | K22 |
| **IleRS** | $^{601}$KMSK$^{604}$ | K601 |
| | $^{602}$MSKSIGNTVSPQDVMNK$^{618}$ | K604 |
| | $^{626}$LWVASTDYTGQMAVSDEILKR$^{646}$ | K645 |
| | $^{1}$MEK$^{3}$ | N-term |
| | $^{549}$DDEGQKMSK$^{557}$ | K554 |
| **ValRS** | $^{555}$MSKSK$^{559}$ | K557 |
| | $^{558}$SKGNVIDPLDMVDGISLPELLEK$^{580}$ | K559 |
| | $^{590}$LADKIR$^{595}$ | K593 |
| | $^{906}$IENKLANEGFVAR$^{918}$ | K909 |
| | $^{1}$AK$^{2}$ | N-term |
| | $^{1}$AKNIQAIR$^{8}$ | K2 |
| **HisRS** | $^{113}$HERPQKGR$^{120}$ | K118 |
| | $^{359}$LMTNHGGGNFKK$^{370}$ | K369 |
| | $^{370}$KQFAR$^{374}$ | K370 |
| **TyrRS** | $^{235}$FGKTEGGAVWLDPK$^{248}$ | K237 |

[a]PLP-labeled residues are in bold.

### *PLP-labeled peptides of HisRS*

PLP-labeled peptides $H_1$–$H_5$ of 31% inactivated histidyl-tRNA synthetase (13 μM HisRS, incubated 20 min in the presence of 4 mM PLP) corresponded to positions 1–2 (AK), 113–120 (HERPQKGR), 370–374 (KQFAR), 1–8 (AKNIQAIR) and 359–370 (LMTNHGGGNFKK) in the primary structure of HisRS, respectively [47]. The PLP-labeled residues of peptides $H_1$–$H_5$ were Ala-1 (labeled on its α-amino group), Lys-118, Lys-370, Lys-2 and Lys-369, respectively (table I).

### *PLP-labeled peptides of TyrRS*

Only one peptide predominantly labeled with PLP ($Y_1$) was isolated from 61% inactivated tyrosyl-tRNA synthetase (13 μM TyrRS, incubated 20 min in the presence of 4 mM PLP). Peptide $Y_1$ corresponded to the fragment 235–248 (FGKTEGGAVWLDPK) in the primary structure of TyrRS, with PLP covalently attached to Lys-237 (table I) [48].

### *Apparent affinity of PLP for the synthetases in the modification of the various lysines*

Since in most cases the plots of PLP incorporation *versus* the residual activity were not linear, the labeled peptides of synthetases were carefully followed as a function of the PLP concentrations in the inactivation mixtures, in order to possibly distinguish between different types of labeling. For each studied synthetase, the yield of each PLP-labeled peptide at a given PLP concentration was expressed as the molar ratio of the amount of the peptide eluting from the RPLC column to the total amount of synthetase present in the labeling experiment. The data summarized in table II were used to plot the yield of each peptide *versus* the PLP concentration in the incubation assays. Figure 5 shows the saturation curve obtained with peptide $m_1$ of M547. The apparent equilibrium constant [$K_d$(app)] for the dissociation of the Schiff base between the reagent and a given lysine residue can be estimated from least square analysis of such a saturation curve.

The $K_d$(app) values associated with each modified lysine were measured with the five studied enzymes.

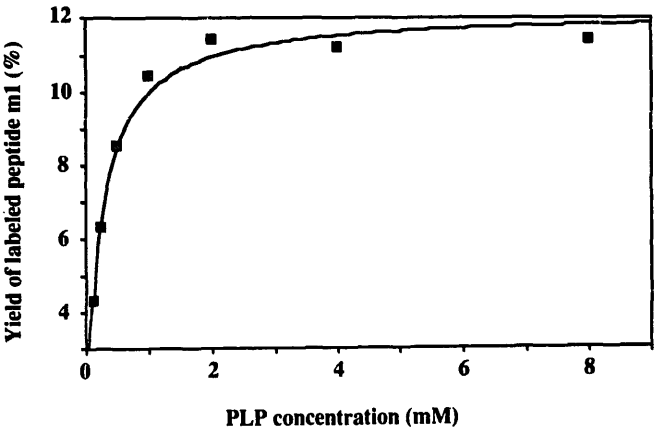


**Fig 5.** Apparent equilibrium constant [$K_d$(app)] for the dissociation of the Schiff base between PLP and lysine-335 (peptide $m_1$) of M547. The yield of the PLP-labeled peptide $m_1$ of M547 was plotted *versus* the PLP concentration in the incubation assay (see also table II). $K_d$(app) represents the PLP concentration corresponding to the half-maximal amount of PLP-labeled peptide $m_1$. The $K_d$(app) value estimated from a least square analysis of the saturation curve is equal to 0.23 ± 0.04 mM.

**Table II.** Variation of the amount of PLP-labeled peptides as a function of PLP concentration. The yield of each peptide is expressed as the percent ratio of the molar amount of the peptide eluting from the RPLC column to the total molar amount of synthetase initially exposed to the labeling. The overall yield of PLP-labeled peptides recovered from the RPLC column depends, for a given synthetase, on : i) the number of mol of PLP incorporated per mol of enzyme; and ii) the yield of recovery of individual labeled peptides from the column, which might reflect the amino acid composition of these peptides. The yield of the only labeled peptide of Try was calculated taking into account that this synthetase sample contained 50% contaminating $MetRS_N$, as deduced from HPLC anlysis of the enzyme as well as automated Edman degradation of PLP-labeled tryptic peptides.

| | *PLP Concentration (mM)* | | | | | | |
|---|---|---|---|---|---|---|---|
| *Enzyme (modified residue)* | *0.10* | *0.25* | *0.50* | *1.00* | *2.00* | *4.00* | *8.00* |
| *HisRS* | | | | | | | |
| K118 | 1.4 | 2.7 | 4.7 | 7.5 | 10.0 | 12.3 | 13.5 |
| K369 | 2.6 | 4.5 | 6.6 | 9.1 | 10.6 | 10.9 | 10.2 |
| K2 | 1.7 | 2.8 | 4.0 | 5.4 | 6.4 | 7.3 | 8.1 |
| alpha amino | 1.2 | 2.6 | 3.9 | 5.9 | 5.9 | 5.6 | 5.8 |
| K370 | 0.7 | 1.2 | 1.9 | 2.5 | 3.3 | 3.3 | 3.3 |
| *M547* | | | | | | | |
| alpha amino | 1.9 | 4.0 | 7.1 | 15.3 | 24.6 | 29.9 | 52.2 |
| K132 | 0.5 | 0.9 | 1.6 | 3.6 | 5.8 | 9.9 | 17.9 |
| K335 | 4.3 | 6.3 | 8.5 | 10.4 | 11.4 | 11.2 | 11.4 |
| K332 | 2.5 | 4.2 | 5.7 | 6.9 | 8.2 | 8.2 | 8.9 |
| K402 | 2.1 | 4.0 | 6.7 | 9.4 | 10.2 | 10.0 | 8.9 |
| *$MetRS_N$* | | | | | | | |
| alpha amino | 1.6 | 3.6 | 6.4 | 12.7 | 21.7 | 31.9 | 42.0 |
| K596 | 5.5 | 10.7 | 16.2 | 22.7 | 26.6 | 28.7 | 31.7 |
| K402 | 2.6 | 5.5 | 8.8 | 13.9 | 15.0 | 16.0 | 21.6 |
| K132 | 0.6 | 1.6 | 2.7 | 5.3 | 8.5 | 13.4 | 19.6 |
| K465 | 0.9 | 1.8 | 3.2 | 5.3 | 5.9 | 9.0 | 12.1 |
| K640 | 1.8 | 3.5 | 3.9 | 4.0 | 8.0 | 13.3 | 11.5 |
| K335 | 3.3 | 5.5 | 8.6 | 9.8 | 10.5 | 10.1 | 9.2 |
| K332 | 1.9 | 3.5 | 4.9 | 6.4 | 6.9 | 7.4 | 6.8 |
| *IleRS* | | | | | | | |
| K22+K117 | 0.8 | 2.2 | 2.9 | 4.8 | 6.8 | 9.9 | 15.4 |
| alpha amino | 1.7 | 2.9 | 5.0 | 6.7 | 8.5 | 9.5 | 9.5 |
| K601 | 3.5 | 5.6 | 8.1 | 9.6 | 10.8 | 10.9 | 9.1 |
| K604 | 2.9 | 4.5 | 6.0 | 6.8 | 7.5 | 8.4 | 9.1 |
| K645 | 1.1 | 1.9 | 3.5 | 5.5 | 5.7 | 6.7 | 7.1 |
| *ValRS* | | | | | | | |
| K909 | 1.1 | 2.9 | 5.2 | 11.1 | 16.3 | 27.3 | 34.4 |
| alpha amino | 2.2 | 4.9 | 7.9 | 16.1 | 20.3 | 26.2 | 23.0 |
| K593 | 0.7 | 1.6 | 2.7 | 5.7 | 7.7 | 12.4 | 15.2 |
| K557 | 3.8 | 7.8 | 11.0 | 14.9 | 14.4 | 15.3 | 11.8 |
| K559 | 1.8 | 3.6 | 4.9 | 6.5 | 5.8 | 7.0 | 6.5 |
| K554 | 1.3 | 2.6 | 3.6 | 5.0 | 5.3 | 6.4 | 6.3 |
| *TyrRS* | | | | | | | |
| K237 | 6.3 | 10.5 | 14.7 | 19.2 | 22.2 | 21.3 | 19.2 |

They ranged from 0.16 to 10 mM (table III). Low $K_d$(app) values indicate lysines labeled at low PLP concentrations. Consequently, these residues were assumed to be the most reactive ones towards the reagent.

## Discussion

Covalent attachment of PLP in the presence of sodium borohydride resulted in a loss of the aminoacid-dependent isotopic [$^{32}$P]PP$_i$-ATP exchange activity of isoleucyl-, methionyl-, valyl-, histidyl- and tyrosyl-tRNA synthetases. In agreement with the present study, Piszkiewicz *et al* [28] previously described the labeling of IleRS, LeuRS and ValRS with PLP. In particular, these authors reported that one mol of IleRS incorporated 1 mol of PLP, concomitantly with 100% enzyme inactivation, and concluded that the reagent was a specific label of the active site of this synthetase. However, the stoichiometries of labeling of IleRS with PLP concentrations greater than 1 mM were not considered.

As shown in figure 2, the inactivation plots at PLP concentrations below 1 mM extrapolate at stoichiometries of 1 mol PLP incorporated per mol of either IleRS, M547 or ValRS, and of 2.4–2.8 mol of PLP incorporated per mol of TyrRS or HisRS. At higher PLP concentrations, the reacting sites became more accessible to the reagent, as illustrated by the 4–5 MetRS : PLP stoichiometry reached at 16 mM PLP.

In the cases of HisRS and TyrRS, however, a unique class of PLP reacting sites seemed to be involved. Extrapolation at 100% inactivation indicated 2.8 ± 0.1 and 2.4 ± 0.1 PLP molecules incorporated per molecule of enzyme, respectively. Considering that these enzymes are dimeric, these stoichiometries are compatible with the incorporation of the label at one site, within or very close to the active center.

The reaction with PLP of a particular lysyl residue in a protein is probably driven by an abnormally low value of the p$K_a$ of its ε-amino group. Actually, the formation of a Schiff base strongly depends on the nucleophilicity of the reacting amino group, which is requested to be unprotonated. Low p$K_a$ values result in a greater mol fraction in the reactive, unprotonated form of the ε-amino group, and, consequently, in a higher rate of reaction. Edsall and Wyman [49] explained that the lowering of the p$K_a$ value of a lysyl residue may be attributed to the location of this residue near other cationic groups in the protein structure. It is worthwhile noting that most of the labeled lysine residues of the studied synthetases are nearly adjacent to other lysine or arginine residues in the primary structure of these synthetases. By following the rate of inactivation of IleRS by PLP as a function of pH, Piszkiewicz *et al* [28] determined an apparent p$K$ value of 8. This value of p$K$ which is 1.4 to 2.6 units lower than expected for the ε-amino group of a free lysine residue [49], may account for the increased reactivity of those lysine residues in the enzymes labeled with PLP. In this context,

**Table III.** Apparent equilibrium constant [$K_d$(app)] for the dissociation of the Schiff base between PLP and a lysine residue. The data from table II were used to plot the yield of each peptide *versus* the PLP concentrations. Values (mM) for $K_d$(app) (the PLP concentration corresponding to the half-maximal amount of a given PLP-labeled peptide), were estimated from least square analyses of the saturation curves obtained (see also fig 5).

| | $K_d$(app) values |
|---|---|
| *IleRS* | |
| K601 | 0.24 ± 0.03 |
| K604 | 0.25 ± 0.03 |
| alpha amino | 0.70 ± 0.1 |
| K645 | 0.80 ± 0.1 |
| K22+K117 | 2.20 ± 0.3 |
| *ValRS* | |
| K557 | 0.31 ± 0.05 |
| K559 | 0.33 ± 0.04 |
| K554 | 0.49 ± 0.04 |
| alpha amino | 1.50 ± 0.1 |
| K593 | 3.50 ± 0.7 |
| K909 | 5.90 ± 0.9 |
| *TyrRS* | |
| K237 | 0.30 ± 0.06 |
| *HisRS* | |
| K370 | 0.16 ± 0.03 |
| K369 | 0.41 ± 0.05 |
| K2 | 0.49 ± 0.05 |
| alpha amino | 1.10 ± 0.4 |
| K118 | 1.20 ± 0.2 |
| *M547* | |
| K335 | 0.23 ± 0.04 |
| K332 | 0.32 ± 0.04 |
| K402 | 0.46 ± 0.06 |
| alpha amino | 4.50 ± 0.5 |
| K132 | 10.00 ± 3 |
| *MetRS$_N$* | |
| K335 | 0.25 ± 0.03 |
| K332 | 0.35 ± 0.05 |
| K596 | 0.60 ± 0.09 |
| K402 | 0.80 ± 0.1 |
| K465 | 1.60 ± 0.2 |
| alpha amino | 4.90 ± 0.8 |
| K132 | 6.00 ± 1 |

the relatively low $pK_a$ of $\alpha$-$NH_2$ groups (9.4–9.7) of proteins may partially explain the systematical labeling of the N-terminal residues of the synthetases studied here.

In order to distinguish the most reactive lysines from the less reactive ones, the labeling of each synthetase was carried out using variable concentrations of PLP, and the tryptic peptides carrying PLP were analyzed (table II). The yield of each PLP-labeled peptide, expressed as the ratio of the amount of this peptide recovered from the RPLC column to the total amount of synthetase present in the labeling experiment, was plotted *versus* increasing PLP concentrations. From the saturation curves obtained, we estimated the apparent equilibrium constant [$K_d$(app)] for the dissociation of the Schiff base between the reagent and a given lysine residue. An important discrepancy was observed between [$K_d$(app)] values of distinct lysines (0.16 to 10 mM ; table III).

Lysine residues with $K_d$(app) values inferior to or equal to 1 mM are lysines 335, 402 and 332 of M547 as well as lysines 335, 402, 332 and 596 of $MetRS_N$; Ser-1 (labeled on its $\alpha$-$NH_2$), lysines 601, 604 and 645 of IleRS; lysines 557, 554 and 559 of ValRS; lysines 369, 370 and 2 of HisRS; and lysine 237 of TyrRS. Considering the results of previous affinity labeling studies [5, 6, 8, 9], it is probable that, with the exception of the N-terminal residues, most of the above cited lysine residues are very close to phosphate binding sites of ATP (or tRNA) on the synthetases. Indeed, among the PLP-labeled lysine residues of MetRS, three (lysines 332, 335 and 402) were previously shown to be critical for activity. Lys-335, part of the Lys-Met-Ser-Lys-Ser (KMSKS) signature sequence [13], was identified as an essential residue in the active center of MetRS [5, 8, 9, 16]. Lys-332 of the signature sequence also makes part of the MetRS active center [50]. Although Lys-402 appears to not be located in the active site crevice of MetRS [50], the affinity labeling of this residue with adenosine triphosphopyridoxal was shown to block enzyme activity [9]. Lysine-557 of ValRS, part of the Lys-Met-Ser-Lys-Ser signature sequence [13] was also shown, together with lysines 593 and 909, to be attached to adenosine triphosphopyridoxal, with concomittent loss of enzyme activity. Thus, labeling with PLP of lysines 332, 335 and 402 of MetRS, or of lysines-554, -557 and -559 of ValRS, could be expected to cause the blockage of the activity of these synthetases. It should be noted that lysines 332, 335 and 402 of 90% inactivated $MetRS_N$ represent altogether only 33.5% of the label attached to the protein. This may be explained by a low yield of recovery of the corresponding labeled peptides from the column.

The only PLP-labeled lysine of TyrRS (Lys-237 of peptide $Y_1$) was previously shown to be affinity-labeled along with Lys-229 and Lys-234 by the oxidized 3'-adenosine of $tRNA^{Tyr}$, suggesting that this residue might be involved in the binding and the guiding of the CCA-arm of $tRNA^{Tyr}$ [6]. Lysines 229, 234 and 237 of *E coli* TyrRS are found conserved as Lys-225, Lys-230 and Lys-233 in the homologous enzyme from *Bacillus stearothermophilus*. Two of these lysines (Lys-230 and Lys-233) belonging to the KMSKS-like sequence KFGKT of TyrRS [6] have been shown by mutagenesis to be involved in the stabilization of the transition state during the synthesis of tyrosyl-adenylate [15]. Thus, labeling of Lys-237 with PLP could be expected to cause the inactivation of *E coli* TyrRS. The reaction of Lys-237 of *E coli* TyrRS with PLP suggests that, similarly to the corresponding lysines of *E coli* MetRS (Lys-335) and of *Bacillus stearothermophilus* TyrRS (Lys-233), Lys-237 might contribute to the catalysis by interacting with one phosphate group of ATP.

Even though lysines 2, 369 and 370 of HisRS show high reactivity with regard to PLP, and may represent phosphate-binding site of ATP or tRNA on this synthetase, these residues do not belong to the sequence motifs characteristic of the 'class 2' aminoacyl-tRNA synthetases.

Residues with $K_d$(app) values equal or superior to 1 mM are Thr-1 (labeled on its $\alpha$-$NH_2$) and Lys-132 of M547, as well as Thr-1 (labeled on its $\alpha$-$NH_2$) and Lys-465 of native MetRS, Lys-22 and -117 of IleRS, Lys-593 and -909 of ValRS, and Ala-1 (labeled on its $\alpha$-$NH_2$) and Lys-118 of HisRS, in addition to unidentified minorily labeled residues of all these enzymes. These residues were supposed to be less reactive toward PLP. Nonetheless, some of them were already shown to be critical for enzyme activity or substrate binding. Previous affinity labeling of lysines 593 and 909 of ValRS with $AP_3$-PL [9] already indicated that these residues were directed toward an ATP or phosphate-binding site. Leon and Schulman [10] previously reported that Lys-465 of MetRS was cross-linked to a dithiobis (succinimidylpropionate)/-propane-1,3-diamine (DTSP/PDA) group attached to cytidine 34 in the anticodon loop of $tRNA^{fMet}$. In addition, it should be noted that Lys-465 lies close to Trp-461 which was shown to play a crucial role in the recognition of $tRNA^{Met}$ (through its anticodon) by methionyl-tRNA synthetase [51]. Labeling of Lys-465 with PLP further indicates that this residue may represent a binding site for a phosphate group of tRNA.

Interestingly, Lys-118 of HisRS belongs to one of the three conserved motifs characteristic of the class 2 synthetases, the RHERPQK sequence commonly referred to as motif number 2. It should be recalled that motifs 2 and 3 participate to the active site of

*E coli* SerRS and *S cerevisiae* AspRS, while motif 1 is located at the contact region between subunits within the dimeric structures [19, 52]. The particular reaction of Lys-118 of HisRS with PLP suggests the involvement of this residue in the binding of a phosphate group of either ATP or tRNA, as expected from the crystallographic structures of *E coli* SerRS and *S cerevisiae* AspRS [19, 52].

To conclude, whatever the studied synthetase, the activity appears sensitive to the presence of rather small PLP concentrations. As discussed above, this behaviour is probably governed by the p*K* values of a few lysine residues as imposed by their location in the 1-D and 3-D synthetase structures. In this context, it is interesting to note that, in figure 2, the initial slope of any plot of PLP incorporation *versus* enzyme inactivation extrapolates to stoichiometries, in good agreement with the known active stoichiometries of the studied synthetases. However, while one bound PLP molecule is statistically enough to account for the complete inactivation of one synthetase molecule, the samples of enzymes inactivated at the lowest PLP concentration contain several discrete peptides labeled in comparable amounts. One must imagine that, when bound, the reagent has the possibility to react with one out of several surrounding lysine residues so that the reaction occurs in a mutually exclusive fashion, and that the modification of any of these lysines is enough *per se* to destroy the activity of the enzyme. This behaviour may be explained in the context of the hypothesis that the active site of synthetases contains several clustered cationic residues. Such an organization may account for the high reactivity of these residues in term of apparent p*K*. As soon as one of these residues is modified with PLP and the activity of the enzyme is impaired, the p*K* values of the other residues may increase and, consequently, their reactivity becomes weaker. In addition, the binding of the first PLP molecule may prevent a second molecule to reach the active site.

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