Lysyl-tRNA synthetase from *Escherichia coli* K12

Chromatographic heterogeneity and the *lysU*-gene product

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In contrast with most aminoacyl-tRNA synthetases, the lysyl-tRNA synthetase of *Escherichia coli* is coded for by two genes, the normal *lysS* gene and the inducible *lysU* gene. During its purification from *E. coli* K12, lysyl-tRNA synthetase was monitored by its aminoacylation and adenosine(5')tetraphospho(5')adenosine (Ap₄A) synthesis activities. Ap₄A synthesis was measured by a new assay using DEAE-cellulose filters. The heterogeneity of lysyl-tRNA synthetase (LysRS) was revealed on hydroxyapatite; we focused on the first peak, LysRS1, because of its higher Ap₄A/lysyl-tRNA activity ratio at that stage. Additional differences between LysRS1 and LysRS2 (major peak on hydroxyapatite) were collected. LysRS1 was eluted from phosphocellulose in the presence of the substrates, whereas LysRS2 was not. Phosphocellulose chromatography was used to show the increase of LysRS1 in cells submitted to heat shock. Also, the Mg²⁺ optimum in the Ap₄A-synthesis reaction is much higher for LysRS1. LysRS1 showed a higher thermostability, which was specifically enhanced by Zn²⁺. These results *in vivo* and *in vitro* strongly suggest that LysRS1 is the heat-inducible *lysU*-gene product.

INTRODUCTION

Every eukaryotic and prokaryotic organism tested to date synthesizes a number of heat-shock proteins in response to heat and other forms of stress. In *Escherichia coli* the transient synthesis of 17 proteins is induced by a shift-up in temperature [1] under the control of the *rpoH*-gene product, which is an alternative sigma factor (σ^{32}) necessary for recognizing the heat-shock promoter sequence [2,3]. Seven of the 17 heat-shock proteins have been identified and appear to be involved in major macromolecular processes of the cell, in particular the inducible lysyl-tRNA synthetase, the product of the *lysU* gene [4,5]. Lee *et al.* [6–8] showed that *E. coli* and *Salmonella typhimurium* cells accumulate very rapidly the dinucleoside oligophosphates Ap₄A, Ap₃Gpp, Ap₄G, Ap₃G and Ap₃A after heat shock, ethanol stress and exposure to a variety of oxidizing agents. Their results strongly suggest that these adenylated nucleotides are the sensors of oxidative stresses and that particular ones may be 'alarmones' to specific stresses: Ap₄A for heat and Ap₃Gpp for oxidative damage to amino acid biosynthesis [8]. The recent findings of Grossman and co-workers [9] that the stringent response in *E. coli* induces expression of heat-shock proteins support this view. Denisenko [10] has also reported that Ap₃A levels are elevated by heat shock in yeast. Recently, Baker & Jacobson [11] measured an increased content of adenyl dinucleotides, including Ap₄A, in cultured mammalian cells after several different treatments that induce the synthesis of stress proteins. Ap₄A and the related dinucleoside oligophosphates are synthesized by some aminoacyl-tRNA synthetases by reaction of the aminoacyl-adenylate-enzyme intermediate complex with a second ATP (respectively ppGpp, GTP, GDP, ADP) molecule [12–15]. Micromolar

Zn²⁺ concentrations stimulate the rate of Ap₄A synthesis by *E. coli* alanyl-, lysyl-, phenylalanyl- and prolyl-tRNA synthetases, and out of all of them, lysyl-tRNA synthetase (LysRS) has the highest Ap₄A synthesis activity in the presence of Zn²⁺ [16]. In *E. coli* Ap₄A is catabolized to ADP by Ap₄A pyrophosphohydrolase, which is activated by Co²⁺ ions and strongly inhibited by micromolar Zn²⁺ concentrations [17,18]. The free Zn²⁺ concentration modulates the intracellular concentrations of Ap₄A by strongly activating the biosynthetic enzymes and inhibiting the catabolic pathway.

The way in which these nucleotides trigger the transcription of the heat-shock genes remains to be elucidated. At the present time, in *E. coli*, no Ap₄A-binding protein associated with DNA or with RNA polymerase has been identified. A tight association between an Ap₄A-binding protein and DNA polymerase- α has been demonstrated in several eukaryotic systems [19,20]. Rapaport & Feldman [21] reported that the free form of this Ap₄A-binding protein contains Ap₄A phosphohydrolase activity, whereas the form resolved from DNA polymerase- α contains no such activity.

MATERIALS AND METHODS

[¹⁴C]Lysine and [¹⁴C]ATP were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.), DNAase (RNAase-free) was from Worthington (Freehold, NJ, U.S.A.) and inorganic pyrophosphatase and alkaline phosphatase were from Boehringer (Mannheim, Germany). Unfractionated tRNA from *E. coli* and (NH₄)₂SO₄ (special enzyme grade) were obtained from Schwarz-Mann (Orangeburg, NY 10962, U.S.A.). L-Lysine, Hepes, ATP, bovine serum albumin, strepto-

Abbreviations used: Ap₄A, adenosine(5')tetraphospho(5')adenosine; Ap₃Gpp, adenosine(5')triphospho(5')guanosine 3'-diphosphate; Ap₄G, adenosine (5')tetraphospho(5')guanosine; Ap₃G, adenosine(5')triphospho(5')guanosine; Ap₃A, adenosine(5')triphospho(5')adenosine; ppGpp, guanosine 5'-diphosphate 3'-diphosphate; PEG, poly(ethylene glycol); LysRS, lysyl-tRNA synthetase; HTP, hydroxyapatite; dnaK, the most abundant heat-shock protein in *E. coli* with 5'-nucleotidase activity.

Fig. 1. Retention of Ap_4A on DEAE-cellulose filters

Elution patterns of three superimposed DEAE-cellulose filters loaded with, respectively, 30 μl of reaction mixture containing 100 mM-Hepes, pH 7.6, + 5 mM- MgCl_2 + 400 μM - ZnCl_2 + (a) 5 mM-ATP + 0.1 μCi of [^{14}C]ATP (584 mCi/mmol), or (b) 5 mM- Ap_4A , or (c) 5 mM- Ap_4A + 5 mM-ATP + 0.1 Ci of [^{14}C]ATP, or (d) as (c), but incubated for 30 min at 37 $^\circ\text{C}$ with 2 μl of alkaline phosphatase (7500 units/ml). The filters were eluted with, successively (1) 0.025 M, (2) 0.05 M-, (3) 0.075 M- and (4) 0.1 M- NH_4HCO_3 buffer, pH 7.85. The nucleotide content, measured by A_{260} (—), and radioactivity (----), was plotted against the elution volume.

mycin sulphate and phenylmethanesulphonyl fluoride were from Sigma (St. Louis, MO, U.S.A.). PEG 10000 and the analytical-grade salts were from Merck (Darmstadt, Germany). Nitrocellulose filters (SM 11305) were from Sartorius (Göttingen, Germany). DEAE-cellulose (DE52), phosphocellulose (P11) and DEAE-cellulose filters DE81 were purchased from Whatman (Maidstone, Kent, U.K.). Bio-Rex 70 and hydroxyapatite (Bio-Gel HTP) were from Bio-Rad (Richmond, CA, U.S.A.) and Sephadex G-25 from Pharmacia (Uppsala, Sweden).

The *E. coli* K12 strain and culture conditions were as described previously [22]. Protein concentrations were determined both spectrophotometrically [23] and by the Lowry method [24], as adapted by Polachek & Cabib [25]. SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli [26].

Initial rates of tRNA aminoacylation were measured at 37 $^\circ\text{C}$ with a reaction time of 10 min and an appropriate enzyme concentration. Before the reaction was started, the LysRS was diluted in 10 mM-Hepes, pH 8.0, containing 0.1 mg of serum albumin/ml. The standard reaction mixture, of volume 0.1 ml, contained 100 mM-Hepes, pH 8.0, 5 mM- MgCl_2 , 1 mM-ATP, 10 μM -[^{14}C]-lysine (50 mCi/mmol) and 0.5 mg of unfractionated tRNA.

The kinetic measurements of [^{14}C]lysyl-tRNA formation were corrected for the non-enzymic hydrolysis of the aminoacyl-tRNA, which became an important factor at alkaline pH values and at higher temperatures [27]. Deacylation of [^{14}C]lysyl-tRNA was measured in the standard reaction medium conditions at different pH

values and temperatures. The first-order rate constants (k_b) were calculated from logarithmic plots of the residual [^{14}C]lysyl-tRNA remaining versus time. Plots of k_b versus pH or temperature were linear. These k_b values were used to calculate the initial aminoacylation rate (v_0) by the following equation:

$$v_0 = k_b \cdot [\text{aa-tRNA}] / (1 - e^{(-k_b \cdot t)})$$

in which [aa-tRNA] is the concentration of [^{14}C]lysyl-tRNA at time t . The standard reaction mixture of 0.05 ml for Ap_4A synthesis contained 100 mM Hepes, pH 7.85 + 5 mM- MgCl_2 + 5 mM-ATP + 5 μl of [^{14}C]ATP (593 mCi/mmol; 20 μCi /ml) + 50 μM - ZnCl_2 + 2 μl of inorganic pyrophosphatase (200 units/ml). After the addition of the appropriate quantity of LysRS incubation was performed at 37 $^\circ\text{C}$ for different periods, usually 10 and 20 min. The reaction was stopped by 2 min boiling, followed by cooling on ice. A 2 μl portion of alkaline phosphatase (1500 units/mg; 5 mg/ml) was added and the reaction medium was incubated for 30 min at 37 $^\circ\text{C}$ to destroy the residual ATP. The [^{14}C] Ap_4A was measured by retention on DEAE-cellulose filters [28]. A 30 μl portion of the reaction mixture was pipetted into 1 ml of 0.025 M- NH_4HCO_3 , pH 7.85, on top of three superimposed wetted DE81 filters on a Millipore filtration manifold. After vacuum aspiration the filters were rinsed three times with 3 ml of the same solution. The filters were placed in 5 ml of scintillation fluid (Filter-count from Packard) and, after 15 min of occasional shaking, the radioactivity was measured in a Searle liquid-scintillation counter. A blank without enzyme was always made under identical conditions. The complete

Fig. 2. Elution pattern of hydroxyapatite chromatography

LysRS was measured by [^{14}C]lysyl-tRNA formation ($\square$) and Ap_4A synthesis ($\circ$). Protein was determined by A_{280} (+).

separation of ATP and the quantitative retention of Ap_4A are illustrated in Fig. 1. LysRS was purified by the following procedure where all steps were carried out at 4°C .

Cell disruption

Frozen *E. coli* cells (about 35 g of cell paste) were thawed and resuspended in 50 ml of 0.02 M-potassium phosphate buffer, pH 7.5, containing 2 mM-phenyl-methanesulphonyl fluoride. After addition of a few milligrams of DNAase, the cells were lysed by passage through a French pressure cell. The lysate was centrifuged at 9000 g for 30 min. When necessary the supernatant was diluted to 20 mg of protein/ml.

Streptomycin sulphate precipitation

A 0.2 vol. of 6.2% (w/v) streptomycin sulphate solution was added dropwise to the crude extract with stirring. After 15 min more of stirring, the precipitate was removed by centrifugation for 30 min at 9000 g.

$(\text{NH}_4)_2\text{SO}_4$ fractionation

EDTA (2 mM) was added to the supernatant, which was then fractionated with $(\text{NH}_4)_2\text{SO}_4$. Material precipitating between 40 and 60% saturation was dissolved in 0.05 M-potassium phosphate buffer, pH 7.5, and dialysed overnight against the same buffer.

DEAE-cellulose chromatography

The non-diffusible material was loaded on to a DEAE-cellulose column (2.5 cm $\times$ 50 cm) equilibrated with 0.05 M-potassium phosphate, pH 7.5, at a flow rate of about 2 ml/min. The column was washed with 500 ml of 0.1 M-potassium phosphate, pH 7.5, and eluted by using a phosphate gradient with 500 ml of 0.1 M-potassium phosphate, pH 7.0, in the mixing chamber and 500 ml of 0.4 M-potassium phosphate, pH 6.5, in the reservoir. LysRS activity, monitored by [^{14}C]lysyl-tRNA formation and Ap_4A synthesis, was eluted at about 0.23 M. The most active fractions were pooled and precipitated with 0.5 g of $(\text{NH}_4)_2\text{SO}_4$ /ml in the presence of 2 mM-EDTA. After stirring for 30 min, the precipitate was collected by centrifugation, dissolved in 0.02 M-potassium phosphate, pH 6.5, and the solution was

passed through a Sephadex G-25 column (3.8 cm $\times$ 34 cm) equilibrated with 0.02 M-potassium phosphate, pH 6.5, in order to eliminate the salts.

Bio-Rex-70 chromatography

Fractions containing LysRS activity (eluted in the void volume of the Sephadex G-25 column) were pooled, the pH checked, and adsorbed on to a Bio-Rex-70 column (2.5 cm $\times$ 50 cm) equilibrated in 0.02 M-potassium phosphate, pH 6.5. The column was washed with the same buffer containing 40 μM -L-lysine, 0.5 mM-ATP and 1 mM- MgCl_2 , and was eluted with a linear potassium phosphate gradient with 500 ml of 0.02 M-potassium phosphate, pH 6.5, in the mixing chamber, and 500 ml of 0.4 M-potassium phosphate, pH 7.5, in the reservoir also containing L-lysine, ATP and MgCl_2 . LysRS activity was eluted at about 0.3 M-potassium phosphate in the presence of lysine, ATP and MgCl_2 as described by Hirshfield *et al.* [29]. The active fractions were pooled, concentrated by precipitation with 0.5 g of $(\text{NH}_4)_2\text{SO}_4$ /ml in the presence of 2 mM-EDTA and the buffer changed by Sephadex G-25 chromatography in 0.02 M-potassium phosphate, pH 6.5.

Hydroxyapatite (HTP) chromatography

The fractions containing synthetase activity and eluted in the void volume of the Sephadex G-25 column were loaded on to an HTP column (2.0 cm $\times$ 12 cm) equilibrated in 0.02 M-potassium phosphate, pH 6.5, with a flow rate of 0.5 ml/min. The column was washed extensively with the same buffer and eluted by using a phosphate gradient with 300 ml of 0.02 M-potassium phosphate, pH 6.5, in the mixing chamber and 300 ml of 0.25 M-potassium phosphate, pH 6.75, in the reservoir. LysRS was measured by tRNA-lysylation and Ap_4A -synthesis activities. Several enzymic forms (1 to 5, eluted respectively at 86, 105, 130, 150 and 180 mM-potassium phosphate) were observed (see Fig. 2). The different peaks were each concentrated by precipitating with 0.5 g of $(\text{NH}_4)_2\text{SO}_4$ /ml and thereafter dialysed against 0.1 M-Hepes, pH 8.0, and stored at -20°C after adding glycerol to 50% (v/v). The HTP pattern was very reproducible for total extracts and also for several separated forms.

Table 1. Purification of *E. coli* K12 LysRS

For details, see the Materials and methods section.

Fraction	Total protein (mg)	Enzyme activity				
		(a) Lysyl-tRNA formation		(b) Ap ₄ A synthesis		Activity ratio (b/a)
		Total activity (nkat)	Specific activity (nkat/mg)	Total activity (nkat)	Specific activity (nkat/mg)	
Crude extract	2984	357.0 (100)*	0.12	112.4	0.038	0.32
Streptomycin sulphate precipitation	1964	333.0 (93.3)	0.17	160.3	0.082	0.48
(NH ₄) ₂ SO ₄ fractionation	1208	301.4 (84.4)	0.25	218.6	0.18	0.73
DEAE-cellulose chromatography	258.6	254.6 (71.4)	0.99	298.0	1.15	1.17
Bio-Rex-70 chromatography	36.1	85.9 (24.1)	2.41	269.6	7.47	3.14
Hydroxyapatite chromatography						
Peak 1	1.78	5.30 (1.5)	2.98	40.7	22.86	7.68
Peak 2	5.51	32.68 (9.2)	5.93	123.1	22.34	3.77
Peak 3	2.46	9.22 (2.6)	3.75	29.8	12.11	3.23
Peak 4	1.37	3.00 (0.8)	2.19	7.0	5.11	2.33
Peak 5	0.91	2.32 (0.6)	2.55	7.5	8.24	3.23
Phosphocellulose and DEAE-cellulose chromatography of enzyme 1	0.56	1.07 (0.3)	1.91	3.35	5.98	3.13
Phosphocellulose chromatography of enzyme 2	3.10	11.78 (3.3)	3.80	119.3	38.48	10.13

* Values in parentheses are percentages relative to the crude extract.

Phosphocellulose chromatography

Enzyme form 1. LysRS₁ after HTP chromatography was dialysed against 10 mM-potassium phosphate, pH 6.0, containing 10% glycerol and loaded on to a phosphocellulose column (2 cm × 11 cm) equilibrated in the same buffer. After washing with the initial buffer, the LysRS₁ (LysRS form 1) activity was eluted with the same buffer containing 1 mM-MgCl₂ + 0.5 mM-ATP + 0.04 mM-L-lysine + tRNA (0.5 mg/ml). The pooled active fractions were loaded on to a small DEAE-cellulose column (2 cm × 8 cm) equilibrated with 10 mM-potassium phosphate, pH 7.0, containing 10% glycerol. After washing with the same buffer, protein was eluted with the initial buffer, containing 0.25 M-NaCl, while tRNA remained adsorbed. At this stage the LysRS₁ preparation was stored at -20 °C in 50% glycerol after concentration by PEG and dialysis against 0.1 M-Hepes, pH 8.0. Subsequent elution of the phosphocellulose column with 0.2 M-potassium phosphate, pH 7.0, produced 40% extra LysRS activity corresponding to LysRS₂ contaminating LysRS₁ on HTP. LysRS₁ does not adsorb on to phosphocellulose in 10 mM-potassium phosphate, pH 7.0, in contrast with LysRS₂.

Enzyme form 2. LysRS₂ after HTP was dialysed against 10 mM-potassium phosphate, pH 6.0, containing 10% glycerol, and loaded on to a phosphocellulose column (2.5 cm × 15 cm) equilibrated with the same buffer. After washing with two column volumes of the same buffer, elution with 1 mM-MgCl₂ + 0.5 mM-ATP +

0.04 mM-L-lysine + 0.5 tRNA (mg/ml) in the same buffer liberated about 12% of the LysRS activity put on the column (corresponding to enzyme form 1). A linear gradient in potassium phosphate, pH 7.0, was applied with 200 ml of 20 mM-potassium phosphate, pH 7.0, in the mixing chamber and 200 ml of 0.2 M-potassium phosphate, pH 7.0, in the reservoir. The active fractions were pooled, concentrated by dialysis against 20% PEG, and stored frozen at -20 °C in 0.1 M-Hepes, pH 8.0, containing 50% glycerol.

Enzyme form 3. LysRS₃ after HTP was eluted like LysRS₂ on phosphocellulose.

RESULTS AND DISCUSSION

We have developed a new method that made it easy to perform kinetics of Ap₄A synthesis. The [¹⁴C]Ap₄A was retained on DEAE-cellulose filter discs under conditions where AMP and ADP were eluted. Because ATP could not be separated completely from Ap₄A on the filters, a preliminary incubation with alkaline phosphatase after the Ap₄A synthesis had been stopped was necessary. Even including this alkaline phosphatase step, the method is still easier to perform and quicker than the bioluminescence-based assay [30] or separation by t.l.c. on polyethyleneimine-cellulose [31,32]. The purification procedure is summarized in Table 1. The low recoveries after the final phosphocellulose steps are explained by the important loss of activity occurring during the

Table 2. Effect of heat shock on LysRS₁ synthesis

The distribution of LysRS₁ and LysRS₂ was determined for cells grown into the exponential phase in rich medium and subjected to heat shock in the same medium for different times at 45 °C. After lysis, the total extract was freed of RNA by DEAE-cellulose followed by a Sephadex G-25 gel filtration. After adsorption on to phosphocellulose in 10 mM-potassium phosphate, pH 6.0, LysRS₁ was eluted by addition of the substrates in the same medium and LysRS₂ was eluted with 0.1 M-potassium phosphate, pH 7.0. The enzymic activity was measured by [¹⁴C]lysyl-tRNA formation and expressed per mg of total protein in the cell extract.

Fraction	Time (min) . . .	Enzymic activity of LysRS (pkat) after heat shock at 45 °C				
		0	10	15	20	30
Cell extract		120.6	138.3	149.2	145.3	126.4
DEAE-cellulose		83.8	93.6	92.9	76.3	89.5
Sephadex G-25		78.4	63.5	68.6	83.0	76.3
Phospho(P11-)cellulose						
LysRS ₁		3.9	3.9	5.1	7.3	7.2
LysRS ₂		39.6	32.8	33.2	38.6	36.1

concentration and dialysis of such diluted samples. Indeed diluted LysRS samples (0.3 µg/ml) in 0.1 Hepes, pH 8.0 (or 7.4) at 0 °C, even in the presence of bovine serum albumin (0.1 mg/ml) showed a considerable loss of aminoacylation capacity with a half-life time of 190 min for LysRS₁ and of 120 min for LysRS₂. This can be greatly improved by working on more starting material and including 50 % glycerol in the last purification steps. The chromatographic heterogeneity of LysRS on HPT had already been reported by Rymo *et al.* [33] and Plateau *et al.* [34]. The existence of two different LysRS enzymes in *E. coli* encoded by two genes, *lysS* and *lysU*, was demonstrated by Van Bogelen *et al.* [5]. Plateau *et al.* [34] verified that the three peaks of LysRS activity eluted from their HPT column support Ap₄A synthesis. They further purified the enzyme form eluted at the highest potassium phosphate concentration (190 mM) in order to study Ap₄A synthesis. The higher Ap₄A synthesis/lysyl-tRNA formation ratio exhibited by our enzyme 1 after HPT chromatography compared with the other enzyme fractions (2–5) led us to speculate a possible identification with the *lysU* gene product characterized by two-dimensional gel electrophoresis by Hirshfield and co-workers [4,5]. The SDS/polyacrylamide-gel electrophoretic pattern of enzyme 2 after HTP chromatography revealed one major band; enzyme 1, however, showed several bands. To purify enzyme 1, we applied phosphocellulose chromatography and found that enzyme 1 and 2 behave very distinctly on this column. LysRS₁ did not adsorb on to phosphocellulose in 10 mM-potassium phosphate, pH 7.0, whereas LysRS₂ did. After adsorption at pH 6.0, enzyme 1 could be eluted by addition of the substrates [0.5 mM-ATP + 40 µM-L-lysine + tRNA (0.5 mg/ml) + 1 mM-MgCl₂], whereas form 2 remained bound to the column under these conditions and was eluted by increasing pH and ionic strength (0.2 M-potassium phosphate, pH 7.0). Enzyme 3 showed the same behaviour as enzyme 2 on phosphocellulose. Specific post-translational modifications of LysRS could explain the different enzyme forms. In particular autoaminoacylation, as has been reported for yeast phenylalanyl-tRNA synthetase [35], needs to be investigated. After phosphocellulose chromatography,

Fig. 3. Determination of the optimal Mg²⁺ concentration for Ap₄A synthesis catalysed by *E. coli* LysRS₁ and LysRS₂

The reaction mixture contained, in 0.1 M-Hepes buffer, pH 7.85, 5 mM-ATP, 1.6 mM-L-lysine, 80 µM-ZnCl₂, MgCl₂ (various concentrations) and 290 ng of enzyme 1 (a) or 138 ng of enzyme 2 (b). The initial Ap₄A-synthesis rate is plotted against the total MgCl₂ concentration.

Fig. 4. Effect of pH on the lysyl-tRNA and Ap₄A synthesis activities catalysed by *E. coli* LysRS₁ and LysRS₂.

The initial rate of Ap₄A synthesis was measured in 0.1 M-Hepes at various pH values in the presence of 1.6 mM-L-lysine, 5 mM-ATP, 200 μ M-ZnCl₂ and 6 mM-(●) or 9 mM-(○) MgCl₂, with 290 ng of enzyme 1 (a, ○) or 138 ng of enzyme 2 (b, ●). Aminoacylation rates were measured under conditions standard except for the variable pH, with 4.6 ng of enzyme 1 (□) or 3.4 ng of enzyme 2 (■). Corrections for non-enzymic hydrolysis of [¹⁴C]lysyl-tRNA were made.

LysRS₁ and LysRS₂ showed a single band on SDS/polyacrylamide-gel electrophoresis corresponding to M_r of about 72000, which correlates with the M_r of 140000 for the native dimer determined by gel filtration on Sephadex G-200 [29]. The amount of LysRS₁ increases in cells submitted to heat shock. As illustrated in Table 2, LysRS₁ represents about 10% of total activity in cells grown exponentially at 37 °C. This value increases gradually up to 20%, when the cells are heat-shocked for 20 min at 45 °C. After 20 min no further increase is observed. The induction of the *lysU*-gene product by small molecules of the rich growth medium [29] explains its presence at 37 °C. The dependence of the initial rate of Ap₄A synthesis on the Zn²⁺ concentration was identical for both LysRS₁ and LysRS₂. At the optimal Zn²⁺ concentration for Ap₄A synthesis of 400 μ M, the tRNA lysylation reaction was completely inhibited. The half-maximal rate of Ap₄A synthesis corresponded to about 20 μ M-ZnCl₂. ZnCl₂ at concentrations higher than 400 μ M inhibited the rate of Ap₄A synthesis. From the Dixon plot the inhibition constant of Zn²⁺ in the aminoacylation reaction was calculated to be 28 ± 5 μ M for enzyme 1, and only slightly lower, 22 ± 3 μ M, for enzyme 2. These results for enzyme 2 agree with those of Plateau & Blanquet [15]. Also in agreement with these authors we showed that the addition of unfractionated *E. coli* tRNA did not influence the rate of Ap₄A synthesis. The dependence of the initial rate of Ap₄A synthesis on the MgCl₂ concentration is shown in Fig. 3. A marked difference of Mg²⁺ optimum between both enzymes was revealed: the optimal Mg²⁺ concentration is 1 mM above the ATP concentration (5 mM) for LysRS₂, whereas it is 4 mM in excess for LysRS₁. In a search for more discriminating differences between LysRS₁ and

LysRS₂, pH and temperature studies were performed. The optimal pH for Ap₄A synthesis was at about 7.6 in 0.1 M-Hepes buffer, and around 8.3 for lysyl-tRNA formation for both enzymes, as shown in Fig. 4. Fig. 5 illustrates the behaviour of LysRS₁ and LysRS₂ at different temperatures. LysRS₁ and LysRS₂ showed a temperature optimum at 35 °C for aminoacylation, whereas the optimum for Ap₄A synthesis is about 11 °C higher. At temperatures above 52 °C the residual Ap₄A-synthesis activity of enzyme 1 exceeds that of enzyme 2, which could be due to a greater thermostability of enzyme 1.

When incubated in the presence of Zn²⁺ at temperatures above 45 °C [in 0.1 M-Hepes (pH 8.0)/+ 50% glycerol] LysRS₁ exhibits a slower inactivation than LysRS₂, as shown in Fig. 6. The greater thermostability of LysRS₁ correlates well with the results reported by Hirshfield & Yeh [36] on the *lysU*-compared with the *lysS*-gene product in partially purified extracts. Our heat-inactivation experiments were performed at higher temperatures because of the protective effect of glycerol in our enzyme preparations. Kinetics of thermal inactivation (Fig. 7) illustrate that the presence of Zn²⁺ (400 μ M) protects the aminoacylation activity of LysRS₁, whereas it has no effect on LysRS₂. We verified that Mg²⁺ could not replace Zn²⁺ and mimic its stabilization effect. It is noteworthy that the stabilization effect of Zn²⁺ on LysRS₁ activity exists only at higher temperatures; in contrast, at low temperatures, Zn²⁺ seems to cause destabilization. The lower residual lysyl-tRNA-synthesis activity of LysRS incubated for 10 min from 0 to 30 °C in the presence of Zn²⁺ cannot be explained solely by the Zn²⁺ inhibition effect in the final aminoacylation assay, because the final concentration never

Fig. 5. Effect of changing the reaction temperature (θ) on the lysislation (a) and Ap₄A synthesis (b) activities of *E. coli* LysRS₁ and LysRS₂.

(a) Initial reaction rates of [¹⁴C]lysyl-tRNA formation were measured in the standard conditions with 4.6 ng of enzyme 1 (□) or 3.4 ng of enzyme 2 (■) at different temperatures. The corrections for non-enzymic hydrolysis of lysyl-tRNA were applied. (b) The initial rates of Ap₄A synthesis were measured at different temperatures with 290 ng of enzyme 1 (○) or 138 ng of enzyme 2 (●) in 0.1 M-Hepes, pH 7.85, 5 mM-ATP, 200 μM-ZnCl₂, 1.6 mM-L-lysine and 6 mM- (●) or 9 mM- (○) MgCl₂.

exceeds 2 μM, a concentration causing a maximum of 10% inhibition. A conformational change induced by Zn²⁺ or a rapid aggregation of the enzyme may be involved in this behaviour. Goerlich & Holler [37] mentioned a rapid and reversible aggregation of LysRS in the presence of 7–10 μM-Zn²⁺. Different interactions between the subunits of LysRS₁ or LysRS₂ might explain their different sensitivities towards temperature and Zn²⁺. Our experiments *in vitro* show that Zn²⁺ and high temperatures combine to favour LysRS₁ over LysRS₂ and Ap₄A synthesis over lysislation activity, leading us to identify LysRS₁ with the *lysU*-gene product.

We showed that LysRS catalysed 80% of the total Ap₄A synthesis in cell extracts (freed from RNA by DEAE-cellulose, followed by extensive dialysis). This was determined by comparing the Ap₄A synthesis in the presence of 0.1 mM-lysine and in the presence of a mixture of the 19 other amino acids, all at 0.1 mM. The Zn²⁺ concentration in the reaction mixture was lowered to 100 μM to obtain a concentration that was close to optimal for Ap₄A synthesis by the different aminoacyl-tRNA synthetases [16,37]. The same result was obtained with the extract prepared from heat-shocked cells (20 min at 45 °C in the rich growth medium). Up to now the mechanism of induction of the heat-shock response

Fig. 6. Thermal inactivation of *E. coli* K12 LysRS₁ and LysRS₂.

The residual activity after 10 min incubation was plotted against the incubation temperature (θ) for Lysyl-tRNA formation. A portion (10 μl) of LysRS₁ (27.3 μg/ml, □) or LysRS₂ (24.3 μg/ml, ■) in 0.1 M-Hepes, pH 8.0, + 50% glycerol were incubated for 10 min at the indicated temperature, then diluted respectively 25 or 100 times with ice-cold Hepes buffer (0.01 M, pH 8.0) containing bovine serum albumin (0.1 mg/ml) and assayed immediately for the residual activity at 37 °C. The same experiment was carried out in the presence of 0.4 mM-Zn²⁺ (△, LysRS₁; ▲, LysRS₂) and 0.9 mM-MgCl₂ (LysRS₁, ▽).

and the functions of heat-shock proteins remain largely unknown. Recent experiments have shown that the heat-shock proteins can be induced by increasing the level of protein σ^{32} [38,39]. σ^{32} is an unstable protein with a half-life of 4 min, whose synthesis [38] and/or activity [40] is controlled by dnaK. In that way dnaK is involved in the turn-off of the heat-shock response. By inhibiting the 5'-nucleotidase activity of dnaK [41], Ap₄A might interfere with the control of the σ^{32} level and so modulate the heat-shock response. Ap₄A in the cell is synthesized above all by LysRS. During a severe heat shock, the *LysU*-gene product (LysRS₁) might be necessary to maintain Ap₄A production, when the normal LysRS could be inactivated. Ap₄A is also clearly implicated in the oxidation stress response. Immediately after addition of CdCl₂ or 6-amino-7-chloro-5,8-dioxyquinoline, the level of Ap₄A increases drastically. This suggests that Ap₄A is necessary for, but not sufficient to turn on, the oxidation stress response. Under these conditions the *lysU*-gene product is not induced, which suggests that Ap₄A is synthesized by the normal LysRS or the other aminoacyl-tRNA synthetases. We propose that the signal for Ap₄A synthesis is an increase in cytoplasmic free Zn²⁺ (or Cd²⁺), after Zn²⁺ has dissociated from a membrane-bound compound. The induction of the heat-shock response by ethanol [42] and by alkaline pH shift

Fig. 7. Effect of Zn^{2+} on the time course of thermal inactivation of *E. coli* K12 LysRS₁ and LysRS₂.

LysRS₁ (a) (13.6 μ g/ml in 0.1 M-Hepes, pH 8.0, containing 50% glycerol) or LysRS₂ (b) (10.3 μ g/ml in 0.1 M-Hepes, pH 8.0, containing 4% glycerol) were incubated at 47.3 °C. At various times samples were taken, diluted with ice-cold Hepes buffer (0.01 M, pH 8.0), containing bovine serum albumin (0.1 mg/ml) and immediately assayed for the residual aminoacylation activity at 37 °C. The results are expressed as percentages of the non-incubated activity [100% = 45.2 fmol/s in the absence of Zn^{2+} ($\square$), 15.8 fmol/s in the presence of 400 μ M- Zn^{2+} (Δ) and = 36.7 fmol/s in the presence of 1 mM- $MgCl_2$ (∇)]. The experiments were repeated at lower glycerol concentration (18%) with ($\bullet$) and without ($\times$) 400 μ M- Zn^{2+} . For LysRS₂, 100% = 33.8 fmol/s in the absence of Zn^{2+} ($\blacksquare$), 26.0 fmol/s in the presence of 400 μ M- $ZnCl_2$ ($\blacktriangle$) and 13.3 fmol/s in the presence of 1 mM- $MgCl_2$ ($\blacktriangledown$).

[43] and, to a lesser extent, the induction of *LysU* by L-alanine or L-leucine and their dipeptides [29] led us to suppose a triggering role for the membrane. The addition of alanyl- and leucyl-homopeptides results in an increase in the transfer of extracellular peptides into the cell [44].

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