A Peculiar Property of Aspartyl-tRNA Synthetase from Bakers' Yeast: Chemical Modification of the Protein by the Enzymatically Synthesized Aminoacyl Adenylate†

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ABSTRACT: In the presence of aspartic acid and ATP·$MgCl_2$, aspartyl-tRNA synthetase catalyzes the formation of an aspartyl adenylate poorly bound to the enzyme. After dissociation from the synthetase, the free aspartyl adenylate is able to react with various nucleophilic acceptors present in the incubation mixture; it was shown that the activated aspartic acid residue could be transferred to Tris [tris(hydroxymethyl)aminomethane], free amino acids, and amino groups of the synthetase itself or of other proteins. The phenomenon is abolished in the presence of $tRNA^{Asp}$. In the presence of inorganic pyrophosphatase (to prevent the reversion of aspartyl adenylate synthesis) and in Mes [2-(*N*-morpholino)ethanesulfonic acid] or Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer, two to eight lysine residues can be aspartylated per dimeric aspartyl-tRNA synthetase according to the pH of the incubation mixture and the nature of the buffer present. This property can be used to screen the lysine-containing peptides at the surface of the synthetase or of other proteins. Some structural and kinetic properties of the aspartylated synthetase have been studied and compared to those of the native enzyme. The apparent molecular weight of the subunits increases significantly, and the pH_i of the protein shifts toward more acidic values. Kinetic studies show that the maximal rates of tRNA charging and ATP–PP_i exchange catalyzed by the synthetase are not significantly affected by the modification. The aspartylation essentially affects the tRNA binding sites as well as the interrelation between the two ATP binding sites; the binding of aspartic acid remains unaffected.

Aminoacyl-tRNA synthetases constitute one of the most heterogeneous class of enzymes from the structural and functional points of view. Their M_r values range from 54 000 to 380 000, and besides monomers of M_r 54 000–130 000, there are dimers of the α_2 type (with small or large subunits) and tetramers of the $\alpha_2\beta_2$ or α_4 type [review by Joachimiak & Barciszewski (1980)]. Despite their common tRNA aminoacylation function, other activities have been found associated to some synthetases, for example, synthesis of P^1,P^4-bis(5′-adenosyl) tetraphosphate, a product involved in cellular metabolism [review by Zamecnik (1983)].

This paper is a contribution to the study of aspartyl-tRNA synthetase from yeast, an enzyme of the α_2 type (α M_r 62 500; Lorber et al., 1983a) to which a particular focus is given because it could be crystallized free or complexed to tRNA (Dietrich et al., 1980; Giegé et al., 1981; Lorber et al., 1983b). We describe here a new property of aspartyl-tRNA synthetase linked to the unstability of the enzyme–adenylate complex. In a preliminary report, it was already observed that the incubation of the synthetase in an aminoacylation medium deprived of tRNA led to a covalent incorporation of aspartic acid into the protein (Lorber et al., 1982). Under usual acylation conditions the extent of this incorporation remains low. So far such a phenomenon has not been observed for other synthetases.

In order to elucidate the mechanism of this protein modification and to clarify its possible incidence under physiological conditions, an analysis of the aspartylation process and a study of the aspartylated synthetase were undertaken. In particular, the nature of the amino acid residues of the protein that were aspartylated and the effect of $tRNA^{Asp}$ on this process were studied. It was also shown that aspartyl-tRNA synthetase can promote a covalent attachment of aspartic acid residues to free nucleophilic acceptors. Some physical and kinetic properties of the modified synthetase were examined. The study was facilitated by the establishment of conditions increasing the aspartylation extent of the synthetase. Finally, it was found that other synthetases under particular conditions can behave like aspartyl-tRNA synthetase. An application of aspartylation, or more generally of the reactivity of free aminoacyl adenylates, allowing one to screen the three-dimensional structure of proteins in solution will be discussed.

Experimental Procedures

General. [^{14}C]- and [^{3}H]-L-aspartic acid (200 mCi/mmol and 37 Ci/mmol) and [^{14}C]-L-tyrosine, [^{14}C]-L-phenylalanine, and [^{14}C]ATP (400 mCi/mmol, 400 mCi/mmol, and 360 mCi/mmol) were from the Commissariat à l'Energie Atomique; [γ-^{32}P]ATP (5000 Ci/mmol) was from Amersham; [^{32}P]PP_i (21 Ci/mmol) and Omnifluor were from New England Nuclear, and L-amino acids were from Merck. Modified amino acids, ATP, Hepes,[1] Mes, cacodylate, Pipes, Tris, spermine, and inorganic pyrophosphatase (1 unit catalyzed the formation of 1 μmol of inorganic phosphate/min at 25 °C

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[1] Abbreviations: DNP-amino acid, dinitrophenyl-substituted amino acid (α or ϵ); FDNB, 1-fluoro-2,4-dinitrobenzene; FPLC, fast protein liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; $tRNA_{ox}$, periodate-oxidized tRNA; $tRNA_{-A}$ or $tRNA_{-CCA}$, tRNA deprived of the 3′ accepting A or CCA end.

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and at pH 7.4) were from Sigma; Sephadex G-100 and G-200 superfine were from Pharmacia, and glass-fiber disks GF/c were from Whatman. For liquid scintillation counting an Omnifluor solution was used (4 g/L of toluol) when radioactivity was precipitated on 3MM Whatman paper disks or a Bray's solution (60 g of naphthalene, 4 g of Omnifluor, 100 mL of methanol, 20 mL of ethylene glycol, and dioxane up to 1 L) when radioactivity was measured in aqueous solution. Cellulose and Kieselgel TLC sheets (Polygram CEL 400 and 60F254, respectively) were from Macherey-Nagel; before use, Kieselgel sheets were activated by heating them 10 min at 120 °C.

Enzymes and tRNAs. Aspartyl-tRNA synthetase (EC 6.1.1.12) from bakers' yeast was prepared as described by Lorber et al. (1983a). The specific activity measured in the standard aminoacylation mixture (see below) at 37 °C was 600–800 units/mg of protein; the molar enzyme concentration was determined from the absorbance (A_{280nm} = 0.60 mg·mL^{-1}·cm^{-1}) with a M_r of 125 000. Phenylalanyl-tRNA synthetase (EC 6.1.1.20) was purified as described by Kern et al. (1977); the molar enzyme concentration was determined from the absorbance (A_{280nm} = 1.22 mg·mL^{-1}·cm^{-1}) with a M_r of 270 000.

Brewers' yeast $tRNA^{Asp}$ was prepared according to the procedure of Keith et al. (1971) modified by Dock et al. (1984); its acceptance capacity was 1600 pmol/A_{260nm} unit. Brewers' yeast $tRNA^{Val}$ and $tRNA^{Phe}$ (acceptance capacity 1300–1600 pmol/A_{260nm} unit) were obtained by countercurrent fractionation (Dirheimer & Ebel, 1967) followed by conventional chromatographic techniques. Periodate oxidation of the tRNAs was done in the presence of 2 mM $NaIO_4$ as described by Kern et al. (1972). $tRNA_A^{Asp}$ and $tRNA_{CCA}^{Asp}$ were prepared by the sequential degradation of Uziel & Khym (1969).

$tRNA^{Asp}$ Aminoacylation Reaction. The standard aminoacylation mixture contained 100 mM NaHepes buffer, pH 7.2, 15 mM $MgCl_2$, 10 mM ATP, 0.1 mM [^{14}C]-L-aspartic acid (25 000–70 000 cpm/nmol), 30 mM KCl, 8 mg of unfractionated yeast tRNA/mL, and 1–5 nM aspartyl-tRNA synthetase. The enzyme was diluted in a buffer containing 100 mM NaHepes, pH 7.2, and 10% glycerol. After various incubation times at 30 or 37 °C, 40-μL aliquots were transferred on Whatman paper disks; the disks were immersed and washed 3 times during 15 min in 5% TCA, then twice for 5 min in 95% ethanol, and finally dried and counted by liquid scintillation.

[^{32}P]PP_i–ATP Exchange. The standard isotope-exchange mixture contained 50 mM NaHepes buffer, pH 7.2, 10 mM $MgCl_2$, 2 mM ATP, 2 mM [^{32}P]PP_i (1000–3000 cpm/nmol), 10 mM L-aspartic acid, and 1–5 nM aspartyl-tRNA synthetase; the enzyme was diluted as before. After various incubation times at 30 or 37 °C, 80- or 100-μL aliquots were transferred in 200 μL of a quenching mixture containing 1% (w/v) of acid-washed Norit, 10% perchloric acid, and 0.3 M $NaPP_i$. After filtration of the Norit on glass-fiber disks, the filters were washed with water and ethanol and finally dried. The [^{32}P]ATP synthesized was determined by liquid scintillation.

Aspartyl-tRNA Synthetase Modification Reaction and Isolation of the Aspartylated Enzyme. Standard aspartylation mixtures contained 100 mM NaMes buffer, pH 6.0, or 100 mM NaHepes buffer, pH 7.2 or 8.6, 2 mM ATP, 2 or 3 mM $MgCl_2$, 0.3 mM L-aspartic acid (either cold or ^{14}C labeled with a specific activity of 17 500–70 000 cpm/nmol), 5 or 10 units of inorganic pyrophosphate/mL (in a negligible amount compared to aspartyl-tRNA synthetase), and 4.5 μM aspartyl-tRNA synthetase. After 10 h of incubation at 30 °C the medium was supplemented with fresh reactants (1–2 mM ATP, 1–2 mM $MgCl_2$, and 100 μM L-aspartic acid), and the incubation was continued for another 14 h. A total of 200 μL of the incubation mixture was then filtered on a G-100 Sephadex column (25 cm × 1 cm) equilibrated with 20 mM NaHepes buffer, pH 7.2, and 100 mM KCl. The eluted fractions were tested for radioactivity and tRNA charging activity. The aspartylated synthetase eluted in the dead volume of the column was separated from free reactants; 80–90% of the synthetase activity was recovered. The extent of aspartylation of the synthetase was determined by transferring 5- or 10-μL aliquots to 3MM Whatman paper disks, which were treated as described previously for tRNA aminoacylation.

Aspartylation of Proteins in a Two-Compartment Dialysis System. An equilibrium dialysis system containing eight pairs of compartments separated by a dialysis membrane was used. One compartment contained the aspartyl adenylate generating system; the other one, the protein to be labeled. To increase the diffusion rate through the membrane, the latter was made porous by $ZnCl_2$ treatment; membranes were moistened, immersed for 6 min in a $ZnCl_2$ solution (320 g of salt for 180 mL of H_2O), rinsed 3 times in 1 mM HCl, and extensively washed with water.

Characterization by TLC or Electrophoresis of the Products Formed by Reaction of the Activated Aspartic Acid with Free Nucleophilic Acceptors. The aspartylation mixture was as described above (the specific modifications are indicated in legend of Figures 4–6): after the indicated time intervals of incubation at 30 °C, 2-μL aliquots were transferred to cellulose plastic sheets (when amino acids were labeled or when ATP was ^{14}C labeled) or to Kieselgel plastic sheets (when ATP was γ-^{32}P labeled). Products were analyzed by chromatography (4 h at 4 °C) with methanol/ammonia/water (6/2/1, v/v/v) solvent or by electrophoresis (20 mA and 400 V; 95 min at room temperature) in the presence of pyridine buffer, pH 4.4 (10 mL of pyridine, 20 mL of acetic acid, 75 mL of acetone, and 395 mL of water); the sheets were dried and the radioactive spots revealed by photography with a beta camera (Berthold BF 2809 E).

Molecular Weight and Isoelectric Point Determinations. Molecular weight determinations by SDS gel electrophoresis and by Sephadex G-200 gel filtration were conducted as described by Lorber et al. (1983a). Isoelectric focusing was done under the conditions described by Lorber & Giegé (1983).

Dinitrophenylation of Aspartylated Enzyme. Samples of native and aspartylated enzyme were concentrated on a Speed Vac apparatus (Savant Instruments, Model RT 100) to a volume of about 400 μL in Pyrex glass tubes (4-mm i.d., 80 mm long). Protein was precipitated by addition of 100% TCA (w/v) to yield a final concentration of 10% (w/v). They were spun down for 5 min at 2000 rpm in a bench-type centrifuge. The precipitated protein was washed twice with 200 μL of 10% TCA (w/v). For samples of aspartylated enzyme, the combined TCA supernatants were subjected to scintillation counting to check for the absence of released [^{14}C]aspartic acid. At this stage, samples of enzyme could be used for direct amino acid analysis as described further in order to estimate the number of attached aspartic acid residues.

Each precipitated sample was resuspended in 100 μL of 0.5 M $NaHCO_3$ and 8 M urea to which 100 μL of 20% SDS (w/v) was added; it was then incubated at 60 °C for 30 min until all the precipitated material had completely dissolved. To the resulting solution 10 μL of FDNB solution (0.1 mL/mL in acetone) was added, and the mixture was incubated at 37

°C for 24 h. Another 10 μL of the FDNB solution was added, and the reaction was continued for another 24 h (final volume of samples 130 μL); 140 μL of water and 30 μL of 100% TCA (w/v) were added in order to precipitate, the dinitrophenylated protein. The precipitate was washed twice with 200 μL of 10% TCA (w/v) and hydrolyzed under nitrogen with 200 μL of 6 N HCl for 24 h at 110 °C. The hydrolyzate was dried in vacuum (in the presence of NaOH pellets and P_2O_5) and finally subjected to amino acid analysis on a Durrum D-500 analyzer. Therefore, all above successive treatments were carried out in the same pyrex glass tube to avoid any losses of material.

Fast Protein Liquid Chromatography of the Aspartylation Medium. Analytical liquid chromatography of the aspartylation medium or its individual components was operated on a Pharmacia FPLC system using a strong ion-exchanger MonoQ HR 5/5 column (Pharmacia). The column was equilibrated with 10 mM Tris-HCl, pH 7.5. Samples were loaded onto the column through a 500-μL injection loop after dilution in the starting buffer. After adsorption of the sample and washing with the starting buffer (fractions 1–10), the elution of bound molecules was achieved by a gradient of ionic strength from 0 to 0.5 M KCl. A flow rate of 0.5 or 1.0 mL/min was maintained during the experiments, and fractions of 0.5 mL were collected.

RESULTS

The covalent attachment of aspartic acid to the protein requires the presence of ATP and $MgCl_2$; therefore, it can reasonably be assumed that the aspartyl adenylate is implied in this process.

Evidence for a Weak Interaction between Aspartyl-tRNA Synthetase and Aspartyl Adenylate. Like most aminoacyl-tRNA synthetases, aspartyl-tRNA synthetase catalyzes an amino acid dependent ATP–PP_i exchange in the absence of tRNA (results not shown). For most of them, an enzyme–aminoacyl adenylate complex can easily be isolated by gel filtration of the amino acid activation system containing the synthetase, the cognate amino acid, ATP, and $MgCl_2$ [e.g., Kern et al. (1981)]. Generally, because of the high affinity of aminoacyl adenylates for the synthetases [e.g., Baltzinger et al. (1983)] and when inorganic pyrophosphatase is present (to displace the equilibrium completely toward the end products), the complex can be isolated in a 1:1 (adenylate to site) stoichiometry [e.g., Kern et al. (1981)]. When such an experiment is performed with aspartyl-tRNA synthetase [possessing two tRNA sites (Giegé et al., 1982) and two amino acid activation sites (unpublished results)], the filtration of the aspartic acid activation system through a Sephadex G-100 column results in a poor recovery of labeled amino acid in the enzyme fraction as compared to the amount expected assuming that both sites are saturated by adenylate. In contrast to the complexes isolated under similar conditions for other systems, no significant transfer can be obtained after addition of $tRNA^{Asp}$, suggesting that the aspartic acid present in the enzyme fraction is not in its activated form. This amino acid can be present either in a free state or covalently bound to the enzyme according to the experimental conditions. Typical experiments are represented in Figure 1. When the complete aspartic acid activation system was filtered through a Sephadex G-100 column equilibrated with Tris-HCl buffer at pH 7.5, only about one aspartic acid residue per eight binding sites (13%) was found associated to the enzyme peak; this amino acid was acidoprecipitable with the protein (Figure 1A). When gel filtration was performed in cacodylate buffer (pH 6.0) and in the presence of dithioerythritol, the amino acid to binding

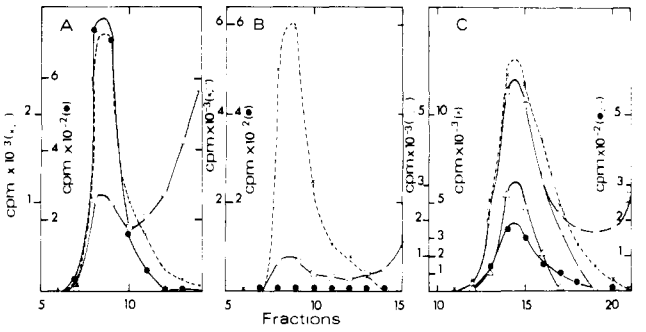

FIGURE 1: Sephadex G-100 filtration of the aspartyl-tRNA synthetase-Asp~AMP complex under various conditions. (A) The column (25 cm × 1 cm) was equilibrated with 50 mM Tris-HCl, pH 7.5, 50 mM KCl, and 5 mM β-mercaptoethanol. The reaction mixture contained 0.1 M Tris-HCl, pH 7.5, 22 μM aspartyl-tRNA synthetase, 100 μM [^{3}H]-L-aspartic acid (sp act. 590 cpm/pmol on Whatman paper in Omnifluor or 2000 cpm/pmol in Bray's liquid scintillator), 0.2 mM ATP, 1 mM $MgCl_2$, and 10 units of inorganic pyrophosphatase/mL. (B) The column (25 cm × 1 cm) was equilibrated with 50 mM cacodylate buffer, pH 6.0, and 0.2 mM dithioerythritol. The mixture contained 0.1 M cacodylate, pH 7.0, 17 μM aspartyl-tRNA synthetase, 125 μM [^{14}C]-L-aspartic acid (225 cpm/pmol), 1 mM ATP, 2 mM $MgCl_2$, and 5 units of inorganic pyrophosphatase/mL. (C) The column (36 cm × 1 cm) was equilibrated with 100 mM cacodylate buffer, pH 6.0. The mixture contained 100 mM cacodylate, pH 6.0, 17 μM aspartyl-tRNA synthetase, 125 μM [^{14}C]-L-aspartic acid (225 cpm/pmol), 1 mM ATP, 2 mM $MgCl_2$, and 5 units of inorganic pyrophosphatase/mL. After 5-min incubation at 30 °C, a 200-μL reaction mixture [4.4 nmol of aspartyl-tRNA synthetase in (A); 3.4 nmol aspartyl-tRNA synthetase in (B) and (C)] was filtered through the column; the flow rate was 10 mL/h, and fractions of 700 [in (A) and (B)] or 580 μL [in (C)] were collected. The activity of aspartyl-tRNA synthetase (×) was tested by diluting 1 μL of each fraction in 50 μL of an aminoacylation mixture, and the [^{14}C]aspartyl-tRNA formed after 10-min incubation at 30 °C was determined in 40-μL aliquots as described under Experimental Procedures. The total radioactivity (□) in each fraction was determined by liquid scintillation of 3-μL aliquots in 5 mL of Bray's scintillator (A) or of 5-μL aliquots (B) or 40-μL aliquots (C) in Omnifluor scintillator. The acidoprecipitable radioactivity (●) in 50- (A), 5- (B), or 40-μL aliquots (C) was determined on Whatman paper disks as described under Experimental Procedures. The transfer of activated aspartic acid (▲) was determined by mixing 40-μL aliquots of each fraction with 25 μL of a transfer medium containing 100 mM NaHepes, pH 7.2, 30 mg/mL unfractionated yeast tRNA, and 30 mM $MgCl_2$; after 10-min incubation at 30 °C, a 50- μL aliquot was analyzed by acidoprecipitation on 3 MM Whatman paper disks. The transfer of the total aspartic acid [activated and free (○)] was determined in a similar way by mixing 40-μL aliquots of each fraction with 25 μL of the transfer medium containing additionally 6 mM ATP.

site ratio was 20%, but aspartic acid did not precipitate with the protein (Figure 1B). Finally, when the column was equilibrated with cacodylate buffer (pH 6.0) but without protector of thiol groups, the amino acid to binding site ratio was 24%, of which 1% of the amino acid coprecipitated with the protein and 7% could be transferred to $tRNA^{Asp}$; therefore, the major part was free and could be transferred to $tRNA^{Asp}$ in the presence of ATP and $MgCl_2$ (Figure 1C). Thus after synthesis by the enzyme, the following are noted: (i) aspartyl adenylate easily dissociates from its site; (ii) only under particular conditions (when the column is equilibrated with cacodylate and in the absence of protectors of thiol groups) it becomes possible to isolate a small part of aspartic acid as aspartyl adenylate; (iii) under other conditions (e.g., in Tris, pH 7.5) the activated aspartic acid reacts with acceptors of the protein to form a stable bond; but when incubated with $tRNA^{Asp}$ this enzyme-bound aspartic acid cannot be transferred to tRNA as evidenced by chromatography on DEAE-cellulose of the incubation mixture (not shown).

Effect of Various Factors on Covalent Incorporation of Aspartic Acid into Aspartyl-tRNA Synthetase: Determination

Table I: Effects of the Nature of Buffer and of pH on Aspartylation of Aspartyl-tRNA Synthetase[a]

buffer	pH	Asp incorporated per dimeric enzyme: per hour (initial rate)	Asp incorporated per dimeric enzyme: after 10 h	establishment of plateau (h)
Tris-HCl	7.5	2.0	2.5	1
	8.0	2.0	1.8	1
	8.8	5.0	4.0	1
	9.6	9.0	6.9	3
Tris-base	11.5	9.0	7.3	3
NaHepes	6.0	2.8	11.6	>10
	7.2	1.5	12.0	>10
	7.8	3.2	13.6	>10
	8.0	3.6	13.8	>10
	8.6	9.8	16.5	>10
NaMes	5.0	1.2	11.0	>10
	5.5	2.9	14.7	>10
	6.0	3.3	15.4	>10
	6.6	1.9	12.7	>10
NaPipes	5.8	2.3	3.2	5
	6.2	1.8	1.9	3
	7.0	0.2	0.7	1
	8.5	0.3	0.5	1

[a] The incubation mixture (200 μL) contained 100 mM buffer at the pH indicated, 2 mM ATP, 3 mM $MgCl_2$, 1 unit of inorganic pyrophosphatase, 0.16 mM [^{14}C]-L-aspartic acid (70 cpm/pmol), and 4.7 μM aspartyl-tRNA synthetase. After various incubation times from 10 min to 10 h at 30 °C, 10-μL aliquots were transferred onto Whatman paper disks, and the radioactivity of the labeled protein was determined as described under Experimental Procedures.

of Optimal Aspartylation Conditions. If the incorporation process involves as intermediate the synthetase-catalyzed aminoacyl adenylate, it should be stimulated by conditions favoring the accumulation of the intermediate. So, the presence of inorganic pyrophosphatase (which cleaves the PP_i and prevents the reversion of aspartic acid activation) stimulates up to 20-fold the incorporation of aspartic acid into the protein. Conversely, the addition of PP_i, by reversing the amino acid activation, decreases it drastically. Inhibition of enzyme aspartylation is also induced by accepting $tRNA^{Asp}$. Since under the experimental conditions used the tRNA was instantaneously charged, it appears that binding of aspartyl-$tRNA^{Asp}$ on the synthetase prevents the aspartylation process. However, nonaccepting tRNA, such as $tRNA_{ox}^{Asp}$, also inhibits the protein aspartylation. The extent of inhibition depends on the degree of saturation of the enzyme by tRNA; at saturation, complete inhibition occurs (not shown). The process is only inhibited to 50% in the presence of noncognate $tRNA_{ox}^{Phe}$ and $tRNA_{ox}^{Val}$ as well as with $tRNA_{-A}^{Asp}$ and $tRNA_{-CCA}^{Asp}$ even when these tRNAs were present at 10 μM (tRNA to binding site = 1:1; not shown). However, the inhibition induced by the two modified tRNAs was reinforced in the presence of adenosine (for $tRNA_{-A}^{Asp}$ and $tRNA_{-CCA}^{Asp}$) or the dinucleotide CpA (for $tRNA_{-CCA}^{Asp}$; not shown).

The aspartylation process occurs without buffers, but they strongly influence it. Buffer and pH effects are summarized in Table I. Among all buffers tested, Tris, Hepes, and Mes are the most efficient. The highest aspartylation extents were obtained in Hepes (pH 8.0–8.6) and in Mes (pH 5.0–6.0). Only low incorporations were observed with Pipes. No significant incorporation occurred with potassium phosphate, cacodylate, and sodium acetate even for pHs that were efficient for other buffers (not shown).

As shown in Figure 2A the kinetics of aspartylation depends upon the buffer concentration. For example, in Tris-HCl (pH 9.6) the initial rates were comparable whatever the buffer concentration (20–400 mM), whereas the plateau values

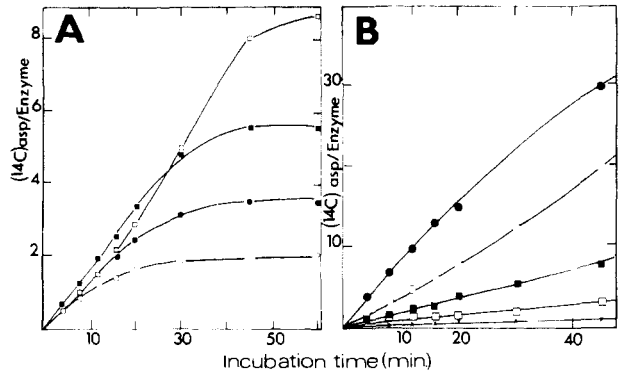

FIGURE 2: Effects of buffer and enzyme concentrations on the aspartylation of aspartyl-tRNA synthetase. The incubation mixtures of a total volume of 100 or 200 μL contained in panel A Tris-HCl buffer, pH 9.6, at 400 (○), 200 (●), 100 (■), and 20 mM (□) and in panel B 100 mM NaHepes buffer, pH 7.8; they contained additionally 2 mM ATP, 3 mM $MgCl_2$, 5 units of inorganic pyrophosphatase/mL, 0.16 mM [^{14}C]-L-aspartic acid (70 cpm/pmol), and 4.5 μM aspartyl-tRNA synthetase in (A) or the following enzyme concentrations in (B): 1.12 (×), 2.25 (□), 4.5 (■), 9 (○), or 22.5 μM (●). At the indicated incubation times at 30 °C, 10-μL aliquots were transferred onto Whatman paper disks, and the radioactivity of the labeled protein was determined as described under Experimental Procedures.

strongly decreased for increasing concentrations. For low concentrations of Tris (up to 40 mM), a lag period was observed. Similar results were obtained with Hepes at pH 7.8 (not shown).

Finally, it was shown that the initial rate of aspartic acid incorporation increases with the enzyme concentration. For low concentrations (up to 9 μM), a lag period occurs that is considerably reduced in the presence of higher enzyme concentrations (Figure 2B).

The previous studies lead to the following optimal aspartylation conditions: 100 mM Hepes buffer, pH 7.2, 2 mM ATP, 2 mM $MgCl_2$, 300 μM aspartic acid, 5 units of inorganic pyrophosphatase/mL, and 4.5 μM aspartyl-tRNA synthetase. To obtain the maximal extent of the reaction, the medium may be supplemented after about 10 h of incubation with fresh reactants: 1–2 mM ATP, 1–2 mM $MgCl_2$, and 100 μM L-aspartic acid. Under these conditions, 12–17 aspartic acid residues could be incorporated per subunit of aspartyl-tRNA synthetase after 24 h of incubation at 30 °C. No transfer of the activated AMP moiety of aspartyl adenylate to protein-accepting groups could be evidenced (not shown).

Physical and Kinetic Properties of Aspartylated Synthetase. The covalent attachment of aspartic acid residues to aspartyl-tRNA synthetase results in a significant increase of the subunits' molecular weights as determined by SDS gel electrophoresis and in a shift of the pH_i of the enzyme toward more acidic values. These increases are a function of the extent of aspartylation. Figure 3A shows that the doublet obtained by SDS gel electrophoresis for the native enzyme also appears for the aspartylated enzyme (electrophoretic properties of aspartyl-tRNA synthetase will be described more extensively in a forthcoming paper); the apparent M_r values of the two bands, which are 65 000 and 63 000 for the native synthetase, became 72 000 and 68 000 after incorporation of 15 aspartic acid residues per subunit. No significant modification of the apparent molecular weight determined under nondenaturing conditions by Sephadex G-200 gel filtration chromatography was observed (M_r 180 000). The pH_i shifts from the 6.3–7.3 domain for the native synthetase (Lorber & Giegé, 1983) to the 4.5–5.5 domain after incorporation of seven residues per subunit (Figure 3B). Extensive aspartylations (when 20 as-

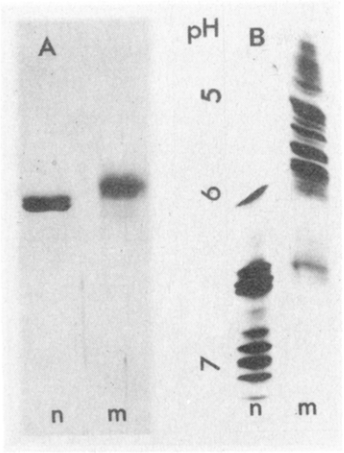

FIGURE 3: Gel electrophoresis of aspartyl-tRNA synthetase either native (n) or modified by aspartylation (m): (A) gel electrophoresis under denaturing conditions in the presence of SDS; (B) isoelectric focusing. The conditions were as described under Experimental Procedures; the extent of aspartic acid incorporation into aspartyl-tRNA synthetase was respectively 15 (A) and 7 (B) residues per subunit.

partic acid residues are incorporated per subunit) are correlated with a slight precipitation of the modified protein, reflecting a decrease in its solubility.

At the plateau of aspartylation, the tRNA acylation activity of the modified synthetase is not considerably affected: for the highest incorporation extents, a decrease of only 20–30% in the activity was detected. To determine whether this decrease reflects a decrease in the rate constant or in the affinity for one or several substrates that become limiting, the K_ms of the native and the aspartylated enzymes for their substrates were determined (Table II). For aspartic acid, it does not vary significantly (120 and 200 μM for the native and the modified synthetases) whereas for $tRNA^{Asp}$ it increases 10–100-fold after incorporation of 15 aspartic acid residues per subunit. As to ATP, a biphasic double-reciprocal plot was obtained for the native enzyme showing two K_m values (40 μM and 1 mM); similar V_{max} values were derived by extrapolation of each phase. For the aspartylated enzyme, this plot is linear, and only one K_m value (20 μM) was determined. Table II shows that the rate constant of $tRNA^{Asp}$ charging remained unaffected. Aspartylation of the enzyme did not affect its ATP–PP_i exchange activity (not shown).

Aspartylation of Free Nucleophilic Acceptors. (A) Reaction with Buffers. When a catalytic amount of aspartyl-tRNA synthetase was incubated in a complete amino acid activation system containing labeled aspartic acid (i.e., under conditions where aspartyl adenylate accumulates without significant reaction with the protein) or when high buffer concentrations were present, aspartic acid could be transferred to the buffer. This was evidenced by analysis of the aspartylation medium by chromatography or electrophoresis, which revealed the formation of new labeled products. Among the buffers tested, Tris was the most efficient acceptor, Hepes was less efficient, and no reaction was observed with cacodylate and potassium phosphate buffers (Figure 4). Increasing concentrations of accepting buffers led to an increase in transfer (not shown). Buffer aspartylation is accompanied with ATP consumption generating AMP and PP_i or P_i (Figure 5) and is stimulated by inorganic pyrophosphatase (Figures 4 and 5).

(B) Reaction with Other Free Acceptors. The presence of free amino acids, spermine, glutathione, or β-mercaptoethanol decreases the rate as well as the extent of aspartic acid incorporation into aspartyl-tRNA synthetase (Table III). The

Table II: Kinetic Parameters of Native and Aspartylated Aspartyl-tRNA Synthetase[a]

	K_m (μM)			turnover (s^{-1})	
enzyme	Asp	ATP	$tRNA^{Asp}$	exptl	cor
native	120 (b)	40 (b) 1200 (b)	0.16 (b)	7.9 (b)	14.3 (b)
	100 (c)	37 (c) 700 (b)	0.06 (c)	7.2 (c)	11.9 (c)
aspartylated	140 (b)	20 (b)	1.2 (b)	7.6 (b)	16.0 (b)
	80 (c)	10 (c)	5 (c)	4.2 (c)	11.8 (c)

[a] The aspartylated synthetase was prepared as described under Experimental Procedures at pH 7.2 (b) or 8.6 (c); 15 and 18 residues were incorporated per subunit of enzyme at pH 7.2 and 8.6 The native synthetase was treated under similar conditions except that the incubation medium was deprived of aspartic acid; tRNA aminoacylation was conducted in 100 mM Hepes, pH 7.2, and 30 mM KCl containing additionally 2 mM ATP, 10 mM $MgCl_2$, 14 μM $tRNA^{Asp}$, and 15–400 μM [^{14}C]-L-aspartic acid (70 cpm/pmol) (for determination of the K_m of aspartic acid), 0.1 mM [^{14}C]-L-aspartic acid (70 cpm/pmol), 10 mM $MgCl_2$, 14 μM $tRNA^{Asp}$, and 0.02–10 mM ATP $MgCl_2$ (for determination of the K_m of ATP), and 0.1 mM [^{3}H]-L-aspartic acid (1000 cpm/pmol), 10 mM ATP, 20 mM $MgCl_2$, and 0.02–1.6 μM $tRNA^{Asp}$ for the native enzyme and 0.2–4 μM for the aspartylated enzyme (for determination of the K_m for $tRNA^{Asp}$); the enzyme concentration was 1–2 nM. The turnover of the $tRNA^{Asp}$ aminoacylation was determined in a medium containing 100 mM Hepes, pH 7.2, 20 mM $MgCl_2$, 10 mM ATP, 5 μM $tRNA^{Asp}$, 200 μM [^{3}H]-L-aspartic acid (125 cpm/pmol), and 1.1 nM native enzyme or 11 nM aspartylated enzymes. After various incubation times at 30 °C, 40- or 80-μL aliquots were transferred onto Whatman paper disks, and the initial rate of $tRNA^{Asp}$ charging was determined as described under Experimental Procedures. Since this apparent turnover was determined in the presence of limiting substrate concentrations, it was corrected according to the K_m values for saturating substrate concentrations. For the K_m determinations, the results were analyzed according to the Lineweaver and Burk representation (1934). When ATP was the variable substrate, the double-reciprocal plots were biphasic in the presence of native enzyme, allowing the determination of two K_m values, similar V_{max} values were determined for each phase.

amino acid effect cannot be attributed to contaminating aspartic acid, since these amino acids at a 1 mM concentration do not affect charging of $tRNA^{Asp}$. Amino acids with blocked α-NH_2 groups are without effect. Among the natural amino acids, cysteine and lysine are the strongest inhibitors. Their inhibition ability is suppressed when their nucleophilic groups are blocked (Table III).

Table IV shows buffer and pH effects on the inhibition by free acceptors on the synthetase modification process. Except for cysteine and glutathione, no significant inhibition occurred in Mes at pH 5.0; a slight inhibition was observed at pH 6.0. In Hepes, inhibition increased by increasing the pH from 7.2 to 8.6. A similar effect was observed in Tris between pH 8.0 and pH 11.5. Significant inhibitions also occurred without buffer.

Figure 6 shows the appearance of the addition product of aspartic acid on other free amino acids present in the medium as evidenced by the fact that the same labeled spot appears whether the radioactivity is carried by aspartic acid or the other amino acid. A similar result was obtained with spermine (not shown).

When the aspartylation mixture containing labeled aspartic acid was analyzed by FPLC, radioactive compounds appeared additionally to the synthetase; two eluted with ATP and AMP and likely correspond to the addition of aspartic acid on the 2′- or 3′-OH of the nucleotide ribose moieties (Figure 7). Aspartylated ADP could be characterized when the synthetase was poorly aspartylated and its elution peak slightly displaced. Free amino acid was not retained by the column and eluted as a sharp peak generally followed by a smaller one. The presence of high buffer concentrations in the medium resulted in a broadening of the second peak, indicating that it corresponds to aspartylated buffer. With β-mercaptoethanol, a new

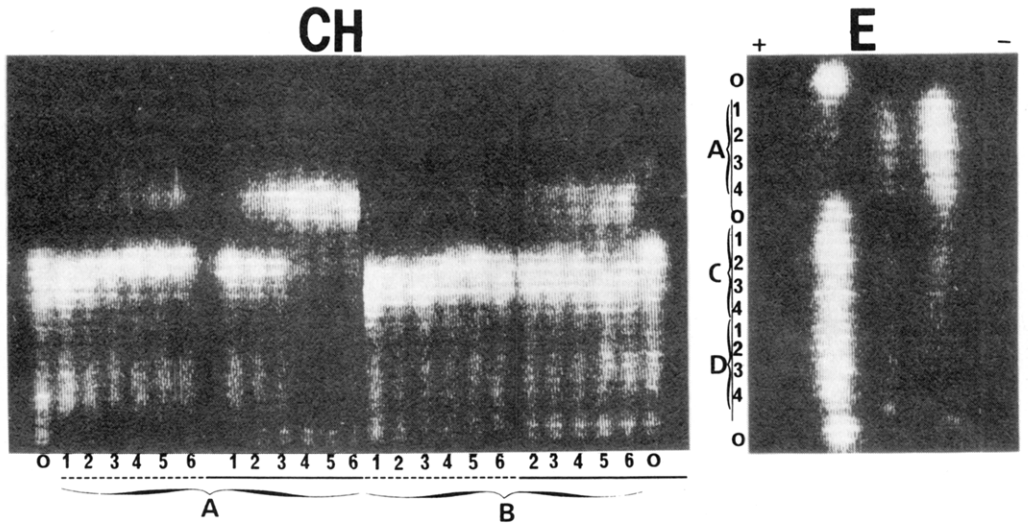

FIGURE 4: Transfer of activated aspartic acid on buffers. The aspartylation mixture was as described under Experimental Procedures and analyzed by chromatography (CH) or electrophoresis (E). It contained as buffer either Tris-HCl, pH 7.5 [(A) 100 mM in CH, 200 mM in E], NaHepes, pH 7.2 [100 mM in (B)], potassium phosphate, pH 7.2 [200 mM in (C)], or Nacacodylate, pH 7.0 [200 mM in (D)]. The experiments were effected either in the absence (––) or in the presence of 10 units/mL (—) inorganic pyrophosphatase. Aliquots of 2 μL were analyzed after various incubation times at 30 °C: in CH at t = 0 (1), 45 min (2), 2 h (3), 4 h (4), 7.5 h (5), and 11 h (6); in E at t = 3 h (1), 7 h (2), 18 h (3), and 29 h (4). (0) Free aspartic acid. The sheets were revealed as described under Experimental Procedures.

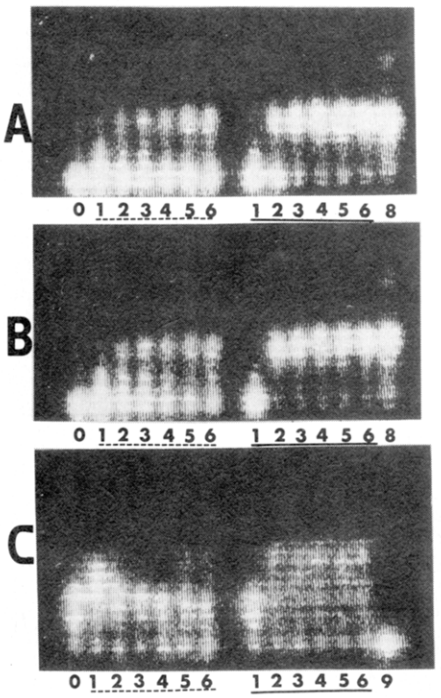

FIGURE 5: Behavior of ^{14}C- and γ-^{32}P-labeled ATP in the course of aspartylation. The aspartylation mixture was as described under Experimental Procedures and contained either 100 mM NaHepes, pH 7.2 (A), 100 mM Tris-HCl, pH 7.5 (B), or 300 mM Tris-HCl, pH 7.5 (C), either [^{14}C]ATP [in (A) and (B)] or [γ-^{32}P]ATP [in (C)], and either no (––) or 5 units/mL (—) inorganic pyrophosphatase. Aliquots of 2 μL were analyzed by TLC after various incubation times at 30 °C: t = 0 (1), 45 min (2), 2 h (3), 4 h (4), 7.5 h (5), and 11 h (6). (0) ATP; (8) AMP; (9) PP_i. ATP does not migrate on cellulose sheets (A and B) but migrates slightly on Kieselgel sheets (C); the minor not migrating spot observed in (C) corresponds to contaminating PP_i.

Table III: Effect of Concentration of Various Free Acceptors and Effect of Modified Acceptors on Aspartylation of Aspartyl-tRNA Synthetase[a]

acceptor or modified acceptor added		concn (mM)	exptl conditions	extent of aspartylation (%)
none			b, c	100
alanine	F	1, 10, 20	b	83, 33, 20
arginine	F	1, 10, 20	b	59, 11, 4
		1	c	33
	α-B	1	c	92
aspartic acid	α-B	1	c	95
asparagine	F	1	c	45
	α-B	1	c	92
cysteine	F	1	c	4
	α+SH-B	1	c	92
glutamic acid	F	1	c	61
	α-B	1	c	92
glutamine	F	1	c	42
	α-B	1	c	94
lysine	F	1, 10, 20	b	51, 10, 5
		1	c	27
	α-B	1	c	63
	ϵ-B	1	c	51
methionine	F	1	c	42
	α-B	1	c	92
serine	F	1	c	39
	α-B	1	c	92
tyrosine	F	1	c	51
	α-B	1	c	92
spermine		1, 10, 20	b	55, 6, 2
glutathione		1	c	24
β-mercaptoethanol		1	d	21

[a] The aspartylation medium was as described under Experimental Procedures and contained either 100 mM NaHepes, pH 7.2 (b), or 20 mM Tris, pH 11.5 (c), or no buffer (d). The extents of enzyme aspartylation were determined after 2.5 (b) or 3 h. (c and d) Incubation times at 30 °C as described under Experimental Procedures and expressed as the percent of the extent obtained in the absence of added free acceptors. F refers to free L-amino acids and B to blocked residues: N^{α}-t-Boc L-amino acids for α-B, N^{ϵ}-t-Boc-L-lysine for ϵ-B, and N^{α}-t-Boc-S-benzoyl-L-cysteine for α+SH-B.

labeled compound appeared as a result of thiol group aspartylation (Figure 7). No free aspartyl adenylate, able to transfer aspartic acid to $tRNA^{Asp}$ in the absence of ATP, could be characterized.

Characterization of Amino Acid Groups of Aspartyl-tRNA Synthetase Modified by Aspartylation. Amino acid analyses of aspartylated and non-aspartylated synthetase before and after dinitrophenylation are summarized in Table V: they concern experiments carried out at acidic, neutral, or alkaline pH, respectively 6.0, 7.2, and 8.6. Of the three potential dinitrophenylation targets, tyrosine, histidine, and lysine, only the last one reacts with aspartyl adenylate yielding a stable

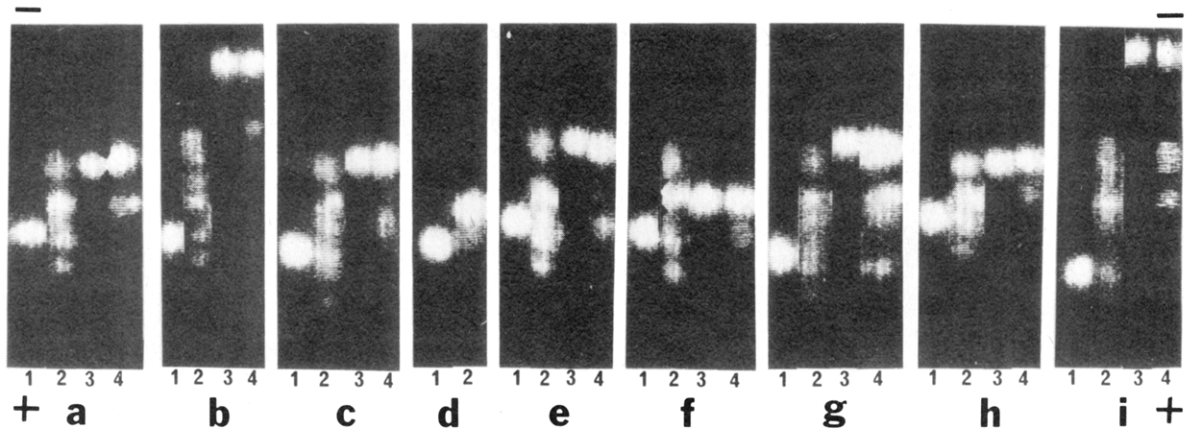

FIGURE 6: Transfer of activated aspartic acid to free amino acids. The incubation mixture was as described under Experimental Procedures and contained 100 mM NaHepes, pH 7.2, 200 μM L-aspartic acid either ^{14}C labeled (35 cpm/pmol) (2) or nonlabeled (4), and 200 (a–f, h, i) or 500 μM (g) another free amino acid either ^{14}C labeled [25 cpm/pmol in (4)] or nonlabeled (2); the amino acids added were (a) alanine, (b) arginine, (c) asparagine, (d) cysteine, (e) glycine, (f) glutamic acid, (g) asparagine, (h) leucine, and (i) lysine. After 18-h incubation at 30 °C, 2-μL aliquots were analyzed on cellulose sheets by electrophoresis as described under Experimental Procedures: (1) aspartic acid; (3) the other free amino acid added in the incubation mixture.

Table IV: Effect of Nature of Buffer and pH on Inhibition of Protein Aspartylation by Various Natural Amino Acids and Glutathione[a]

amino acid or glutathione added	extent of aspartylation in the following buffer						
	Mes at pH		Hepes at pH		Tris at pH		
	5.0	6.0	7.2	8.6	8.0	11.5	none
none	100	100	100	100	100	100	100
alanine	100	93	90	58	84	65	90
arginine	100	84	65	26	49	33	51
asparagine	98	89	74	34	60	48	68
cysteine	12	5	4	3	5	4	3
glutamic acid	88	77	89	57	82	62	79
glutamine	75	86	85	34	61	43	68
glycine	93	88	70	25	50	30	59
histidine	84	75	59	30	52	33	56
leucine	100	96	96	51	76	58	87
lysine	100	91	63	23	45	29	51
methionine	100	93	79	34	60	42	69
proline	100	79	48	13	24	17	40
phenylalanine	96	88	78	32	58	40	63
serine	100	86	76	32	57	42	65
tyrosine	120	75	69	38	62	54	59
glutathione	34	23	15	10	15	25	26

[a] The aspartylation medium was as described under Experimental Procedures and contained 20 mM buffer at the pH as indicated and 1 mM L-amino acid or glutathione. The extent of aspartylation of the aspartyl-tRNA synthetase was determined after 15, 30, 60, and 120 min by acidoprecipitation of an aliquot. The results indicate the percent incorporation after 120 min of incubation.

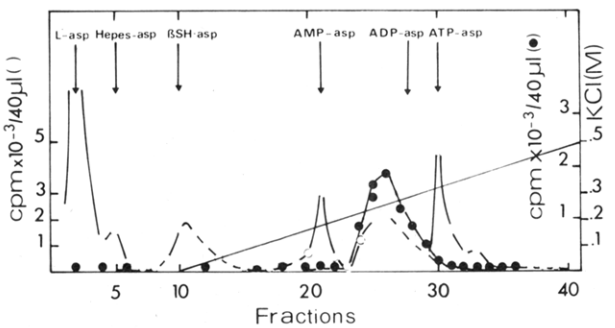

FIGURE 7: Characterization of labeled compounds appearing in the aspartylation mixture containing labeled aspartic acid. The incubation mixture was as described under Experimental Procedures; 20 mM NaHepes, pH 7.2, was present. After 12-h incubation at 30 °C, a 40-μL aliquot was analyzed by FPLC as described under Experimental Procedures. The flow rate was 1 mL/min, and the elution was effected with a linear gradient of KCl (—). The total (O) and the acido-precipitable (●) radioactivities were determined in 40-μL aliquots as described under Experimental Procedures. The elutions of ATP, ADP, and AMP were characterized by chromatography of each of these nucleotides. The elutions of aminoacylated buffer (Hepes-asp) and β-mercaptoethanol (β-SH-asp) were characterized by chromatography of aspartylation mixtures containing 500 mM Hepes or 5 mM β-mercaptoethanol.

peptide-like bond with aspartic acid: indeed two, four, and eight lysine residues are protected by covalent aspartylation of their ϵ-NH_2 group and thus escape dinitrophenylation. The control experiments show that in the native enzyme all tyrosine,

Table V: Amino Acid Composition of Native and Aspartylated Aspartyl-tRNA Synthetase before and after Dinitrophenylation[a]

amino acid	aspartyl-tRNA synthetase							
	before dinitrophenylation				after dinitrophenylation			
	native	aspartylated at			native	aspartylated at		
		pH 6.0	pH 7.2	pH 8.6		pH 6.0	pH 7.2	pH 8.6
Asp	50	57	58	62	52	58	58	56
His	12	12	12	12	0	0	0	0
Lys	42	46	45	46	0.5	2.3	4	8.3
Tyr	19	13	19	18	0	0	0	0

[a] The aspartylated enzyme was obtained by incubation of aspartyl-tRNA synthetase in a complete aspartic acid activation system containing Mes buffer, pH 6.0, or NaHepes buffer, pH 7.2 or 8.6, as described under Experimental Procedures. Measurements of aspartic acid incorporated into the synthetase on aliquots of the incubation mixture after 24 h of incubation at 30 °C indicated the following extents of incorporation: 14–18 Asp/subunit at pH 6.0; 15–20 Asp/subunit at pH 8.2, and 15–20 Asp/subunit at pH 8.6. After isolation of the aspartylated enzymes by gel filtration, the extents of incorporation were respectively 10–13 Asp/subunit, 14–17 Asp/subunit, and 14–17 Asp/subunit for the enzyme modified at pH 6.0, 7.2, and 8.6. For the other amino acids the values obtained for the aspartylated enzyme before and after dinitrophenylation were close to those of the native enzyme: Ala, 42; Arg, 37.5; Cys, 2; Glu, 82; Gly, 33; Ile, 24; Leu, 56; Met, 11; Phe, 28; Pro, 26; Ser, 26; Thr, 27; Try, 2; Val, 28. The results are expressed as moles of residue per mole of subunit on the basis of a M_r of 62 500; each column represents the average of at least two sets of experiments.

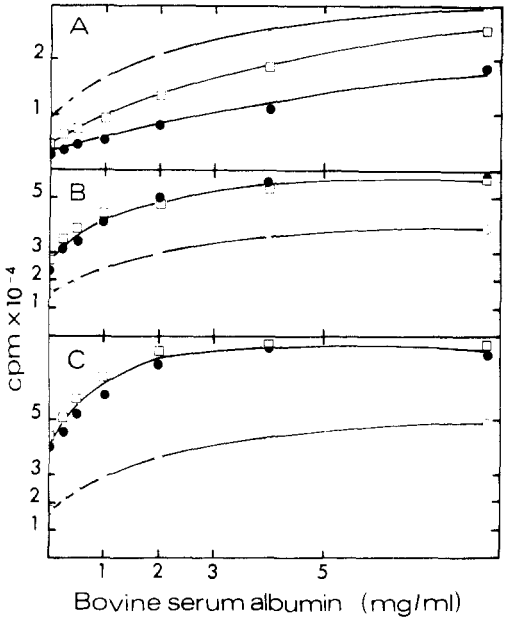

FIGURE 8: Covalent incorporation of aspartic acid into bovine serum albumin. The incubation mixture was as described under Experimental Procedures and contained as buffer either 100 mM Tris-HCl, pH 7.5 (○) or 100 mM NaHepes, pH 7.2 (●) or no buffer but 100 mM KCl (□); the specific activity of aspartic acid was 35 cpm/pmol, and the concentrations of bovine serum albumin were as indicated. After various incubation times [(A) 1 h, (B) 12 h, (C) 25 h] 10-μL aliquots were analyzed by acidoprecipitation as described under Experimental Procedures.

histidine, and virtually all lysine residues are dinitrophenylated. A comparison of direct amino acid analyses prior to dinitrophenylation yields an accurate estimation of the number of aspartic acid residues covalently attached to the enzyme through stable chemical bonds: 7, 8, and 12 residues at pH 6.0, 7.2, and 8.6, respectively. It must be pointed out that radioactivity measurements systematically gave higher values for aspartic acid incorporation. However, no radioactivity was released during the different experimental steps as checked by scintillation counting. The reason for such a discrepancy is as yet unclear.

Another striking feature of the results listed in Table V is the significant increase in the aspartic acid content of the protein as revealed after its dinitrophenylation. This means that the α-NH_2 group of some of the incorporated residues escapes dinitrophenylation. Therefore, such a residue will appear as free aspartic acid in the amino acid analysis (in our analytical system α-DNP and ϵ-DNP amino acids are not detected). Such a result is only possible if the free α-NH_2 group of a covalently attached aspartic acid molecule becomes in turn a new target for another aspartylation reaction, thus giving rise to di- or polyaspartyl side chains bound to the same lysine residue of the protein.

Aspartylation of Other Proteins. When added to the aspartylation medium, other proteins too incorporate aspartic acid covalently. Figure 8 shows the increase of acidoprecipitable aspartic acid in an activation system containing increasing amounts of bovine serum albumin. A limit extent of incorporation is reached for 8 mg of bovine serum albumin/mL. As already observed, the initial rate of protein aspartylation occurs faster in Tris, pH 11.5, than in Hepes, pH 7.2, or without buffer (results obtained after 1-h incubation, Figure 8A). For longer incubation times (12 h, Figure 8B) or at the plateau (after 25 h, Figure 8C), the highest incorporations are obtained in Hepes or without buffer; the extent of incorporation in Tris, pH 11.5, is significantly lower.

Table VI: Covalent Incorporation of Aspartic Acid into Various Proteins As Studied in a Two-Compartment System[a]

expt no.	compartment 1, [^{14}C]Asp/enzyme	compartment 2, [^{14}C]Asp/protein	
	mol/mol	nmol/mg	mol/mol
1	48	125	25
2	40	220	55
3	50	103	16
4	53	85	4
5	52	81	1.2
6	61	95	1.2
7	49	110	7
8	50	64	4.5

[a] Compartments 1 and 2 contained 20 mM NaHepes, pH 7.2, 4 mM ATP, 4 mM $MgCl_2$, 0.4 mM [^{14}C]-L-aspartic acid (17.5 cpm/pmol), and 10 units of inorganic pyrophophatase/mL. Compartment 1 contained 4.5 μM aspartyl-tRNA synthetase, and compartment 2 contained another protein: in 1, 3 μM valyl-tRNA synthetase (0.4 mg/mL); in 2, 2 μM catalase (0.5 mg/mL); in 3, 3.2 μM alcohol dehydrogenase (0.5 mg/mL); in 4, 11 μM ovalbumin (0.5 mg/mL); in 5, 35 μM lysozyme (0.5 mg/mL); in 6, 38 μM cytochrome *c* (0.5 mg/mL); in 7, 8 μM bovine serum albumin (0.5 mg/mL); in 8, 9 μM hemoglobin (0.6 mg/mL). Each compartment contained a volume of 80 μL, and both compartments were separated by a dialysis membrane treated as described under Experimental Procedures. After 40 h of dialysis at 28 °C, the ^{14}C-labeled acidoprecipitable aspartic acid was determined in a 60-μL aliquot as described under Experimental Procedures.

Two approaches can be used to label foreign proteins and to obtain them free from contaminating aspartyl-tRNA synthetase: (i) The protein is incubated together with aspartyl-tRNA synthetase in a complete aspartylation mixture; during incubation both proteins are labeled; they are separated by FPLC. In such a way, several aspartylated proteins could be isolated (e.g., hemoglobin, cytochrome *c*, lysozyme, alcohol dehydrogenase, catalase, ovalbumin, bovine serum albumin, phenylalanyl- and valyl-tRNA synthetases). (ii) The protein is labeled by a two-compartment system separated by a dialysis membrane; one compartment contains the aspartyl adenylate generating system and the other one the protein to be labeled. The results of such experiments are given in Table VI. Approximately similar amounts of aspartic acid are incorporated per milligram of protein present in the second compartment.

Generalization of Protein Aminoacylation: Covalent Attachment of Tyrosine to Phenylalanyl-tRNA Synthetase. It has been shown that phenylalanyl-tRNA synthetase can misactivate tyrosine in the presence of ATP and $MgCl_2$ (Igloi et al., 1978); because of its low affinity, tyrosyl adenylate dissociates from phenylalanyl-tRNA synthetase (Lin et al., 1983). This heterologous activation is analogous to that of aspartic acid; thus, the activated tyrosine should react with acceptors of the protein. Figure 9 shows that, in an activation medium containing inorganic pyrophosphatase, tyrosine is covalently bound to phenylalanyl-tRNA synthetase. The reaction occurs in Mes (pH 6.0), Hepes (pH 7.8), and Tris (pH 8.8) buffers. In the correct system, because of the absence of significant dissociation of phenylalanyl adenylate from the enzyme, no incorporation of phenylalanine occurs.

Discussion

The present paper demonstrates the involvement of aspartyl adenylate in an aspartylation process of acceptors other than tRNA. This phenomenon was up to now not described for other aminoacyl-tRNA synthetases. The reason for that relies on specific properties of aspartyl-tRNA synthetase.

Particular Behavior of Enzyme-Synthesized Aminoacyl Adenylate in the Aspartic Acid System. A total of 17 of the 20 aminoacyl-tRNA synthetases forms an aminoacyl adenylate

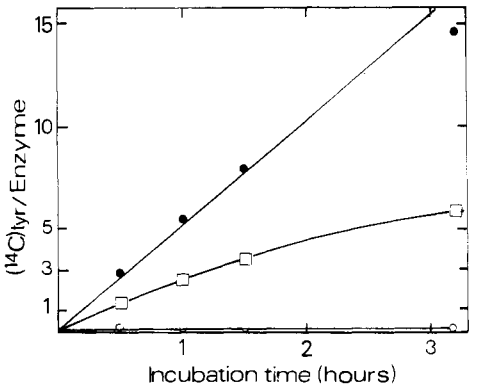

FIGURE 9: Covalent attachment of tyrosine to phenylalanyl-tRNA synthetase. The incubation mixture of a total volume of 50 μL contained 100 mM NaMes, pH 6.0 (○), 100 mM NaHepes, pH 7.8 (●), or 100 mM Tris-HCl, pH 8.8 (□), 0.1 mM [^{14}C]-L-tyrosine (103 cpm/pmol), 2 mM ATP, 3 mM $MgCl_2$, 5 units of inorganic pyrophosphatase/mL, and 4 μM phenylalanyl-tRNA synthetase. After various incubation times at 30 °C, 10-μL aliquots were transferred onto Whatman paper disks, and the radioactivity of the enzyme was determined as described under Experimental Procedures. No incorporation was observed when tyrosine was replaced by phenylalanine.

in the absence of tRNA, and strong arguments for its participation in the overall tRNA charging process have been reported (Fersht & Jakes, 1975; Fersht & Kaethner, 1978; Fasiolo & Fersht, 1978; Mulvey & Fersht, 1978; Kern & Gangloff, 1981). However, for several systems, starting from preformed enzyme–aminoacyl adenylate complex, incomplete transfers of the activated amino acid to the tRNA have been reported despite the high affinity of the intermediate for the synthetase (Kern et al., 1981; Baltzinger et al., 1983). It has been shown that part of the enzyme-bound adenylate hydrolyzes after association of the cognate tRNA (Fersht & Jakes, 1975; Kern et al., 1981). An opposite behavior is found for the aspartic acid system. In that case, the enzyme–aspartyl adenylate complex easily dissociates because of the poor affinity of aspartyl adenylate for the synthetase. The same phenomenon has been reported in the heterologous phenylalanyl-tRNA synthetase activating tyrosine system; after misactivation, tyrosyl adenylate dissociates from the enzyme because of its low affinity (4 nM and 1–2 μM for phenylalanyl and tyrosyl adenylates; Lin et al., 1983). Despite the existence of the amino acid dependent ATP–PP_i exchange activity, it has been shown for both systems that no enzyme–aminoacyl adenylate complex can be isolated under standard conditions (i.e., in the absence of tRNA; Figure 1 and Lin et al., 1983).

Mechanistic Aspects of Aspartylation Process. Activated aspartic acid can be transferred to free or to protein acceptor groups. No transfer of the adenylate moiety occurs. The question can be raised as to whether acceptors react with enzyme-bound or with dissociated aspartyl adenylate. We bring evidence that protein aspartylation is promoted by free aspartyl adenylate: (i) A relationship was established between the poor affinity of aminoacyl adenylates for their synthetases and the ability of the activated amino acid to be transferred to protein acceptor groups; in cognate systems, other than that of aspartic acid, the aminoacyl adenylate is generally strongly bound to its site so that protein aminoacylation was never found. (ii) Since proteins can be aspartylated to a high extent even when separated from the aspartyl adenylate generating system by a dialysis membrane (Table VI), the activated amino acid necessarily dissociates from the enzyme before reacting with the protein acceptors.

Buffers stimulate or hinder protein aspartylation (Table I), suggesting that they act as catalysts. However, since aspartylation can occur even in the absence of buffer, it rather seems that they intervene in the dissociation of aspartyl adenylate from the synthetase. Buffers able to promote protein aspartylation increase the rate of dissociation of the activated amino acid, whereas those unable to promote protein aspartylation decrease it. This is suggested by gel filtration experiments of the aspartic acid activation system conducted with various buffers (Figure 1). Buffers unable to promote protein aspartylation (i.e., cacodylate) allow the isolation of small amounts of enzyme-bound aspartyl adenylate; those promoting aspartylation (i.e., Tris or Hepes) do not allow it. Surprisingly, the most efficient buffers for protein aspartylation also accept the activated aspartic acid. Several reports have shown that Tris can accept activated amino acids [i.e., Schubert & Pinck (1974)]; Hepes too is an acceptor (Figures 4 and 7). Thus it seems that the dissociation of the enzyme-bound aspartyl adenylate promoted by buffers is correlated with the aminoacylation of these buffers. The extent of dissociation depends upon the nature, the pH, and the concentration of the buffer. The most efficient show pH effects on the initial rate of protein aspartylation (Table I); for instance, in Tris this rate increases with pH as the acceptance ability of the buffer increases, reflecting pH-induced modulations in the rate of appearance of free aspartyl adenylate. The buffer concentration probably modulates the rate of dissociation of aspartyl adenylate, since increasing buffer concentrations, despite the increase in the competition to accept the activated amino acid, do not decrease significantly the initial rate of protein aspartylation (Figure 2A).

A lag phase generally indicates the existence in an overall process of two reactions involving a common intermediate and occurring at similar rates (Fersht et al., 1978; Kern & Gangloff, 1981). The turnover of $tRNA^{Asp}$ charging (14 s^{-1}; Table II) and of ATP–PP_i exchange (20 s^{-1}; not shown) is considerably higher than the rate of protein aspartylation; thus, aspartyl adenylate is generated faster by the synthetase than it is further consumed in the aspartylation process. After its fast synthesis (1), the free intermediate appears slowly according to a first-order reaction (2) and then reacts slowly with acceptor groups according to a second-order reaction (3):

$$E + Asp + ATP \underset{fast}{\overset{MgCl_2}{\rightleftharpoons}} E\cdot Asp\sim AMP + PP_i \quad (1)$$

$$E\cdot Asp\sim AMP \underset{slow}{\rightleftharpoons} E + Asp\sim AMP \quad (2)$$

$$Asp\sim AMP + acceptor \xrightarrow[slow]{} acceptor\text{–}Asp + AMP \quad (3)$$

Thus, the lag phase indicates that at the early time of the reaction the low concentration of free aspartyl adenylate only allows a poor aspartylation of the protein. The rate of aspartylation increases as the concentration of free aspartyl adenylate increases until the rates of both reactions are equal and the concentration of the free intermediate reaches a steady-state value. In the presence of high buffer and enzyme concentrations, the steady-state concentration of the intermediate is faster established: the activated amino acid either accumulates or reacts faster at the early time of the reaction.

The modulating effects observed for PP_i and inorganic pyrophosphatase on the extent of the aspartylation process reflect the modulation by these compounds of the concentration of free aspartyl adenylate. This relies to their modulation of the equilibrium of the amino acid activation step (1). For several systems it has been shown that complete saturation of the activation sites by aminoacyl adenylate starting from free amino acid and ATP·$MgCl_2$ only occurs in the presence of

inorganic pyrophosphatase (Fersht et al., 1975; Fasiolo & Fersht, 1978; Kern et al., 1981); this is because inorganic pyrophosphatase hinders the pyrophosphorolysis of aspartyl adenylate.

Inhibition of the Aspartylation Process in the Presence of tRNA^{Asp}. The inhibition of the aspartylation process by tRNA^{Asp} can be interpreted in three ways: (i) by increasing the affinity of PP_i for the synthetase and thus strongly reversing the activation step, the tRNA decreases the concentration of free aminoacyl adenylate; (ii) by modifying the conformation of the protein, tRNA^{Asp} promotes the inaccessibility of the protein-accepting groups; (iii) by increasing the affinity of aspartyl adenylate for the synthetase, tRNA^{Asp} strongly decreases the concentration of the free reactant. The first interpretation can be excluded since the tRNA effect occurs even in the presence of inorganic pyrophosphatase. A protecting effect induced by tRNA against the modification cannot be excluded, but it would be surprising that tRNA^{Asp} protects all residues of the protein able to be modified. Other studies have shown that tRNA^{Asp} increases the affinity of aspartyl adenylate for the synthetase (to be published) and thus favor the last interpretation. The tRNA effect observed here occurs with charged tRNA^{Asp} as well as with nonaccepting $tRNA_{ox}^{Asp}$. Its optimal effect requires the presence of the adenosine end, because $tRNA_{-A}^{Asp}$ and $tRNA_{-CCA}^{Asp}$ have only a poor effect and because their effect is reinforced by adenosine and adenosine or CpA, respectively. Thus, the tRNA effect is, a least partly, promoted by the interaction of the adenosine or the CCA end with the synthetase. Heterologous tRNAs that have a lower affinity for the synthetase than the cognate tRNA (Ebel et al., 1973) are unable to efficiently inhibit the aspartylation process. This agrees with results obtained by other approaches showing that homologous, contrary to heterologous, tRNAs induce conformational changes on synthetases, some of them requiring the adenosine or the CCA end of the tRNA [Kern & Giegé (1979) and references cited therein; Bacha et al., 1982].

Reaction of the Activated Amino Acid with Free Acceptors and Incidence on Protein Aspartylation. Only some buffers (i.e., Tris or Hepes) can accept activated aspartic acid (Figure 4). As already discussed, this acceptance capacity is probably correlated to their ability to promote the dissociation of the enzyme-bound aminoacyl adenylate. The activated aspartic acid can also be transferred to other free acceptors: those involved in the modification process (aspartic acid, ATP, AMP) or any compounds containing accepting groups (i.e., spermine, components-containing thiol groups, ADP, other free amino acids). Related to this observation is the aminoacylation of ATP by tryptophanyl-tRNA synthetase from *Escherichia coli* (Joseph & Muench, 1971) and *Bacillus stearothermophilus* (Coleman & Carter, 1984) after binding of the nucleotide on the site of the tRNA terminal 3′-adenosine.

Cysteine, glutathione, or β-mercaptoethanol, which contain thiol groups, inhibit protein aspartylation and accept aspartic acid in a wide range of pH (Table IV and Figure 6). This is not the case for most amino acids (with α-NH_2 or ϵ-NH_2 lysyl, imidazole histidyl, or hydroxyl tyrosyl accepting groups), which inhibit protein aspartylation at alkaline pH whatever the nature of the buffer present (Table IV); this reflects favorable ionization of these groups to accept the activated amino acid only at alkaline pH. Aspartic acid polymers of variable sizes growing on an initially free acceptor probably are formed as suggested by the large distribution of the labeled spots observed by TLC and electrophoresis of the aspartylation mixture (Figures 4 and 6). The plateau of protein aspartylation corresponds to the complete consumption of the small reactant, amino acid, or ATP, which become limiting; its extent reflects the relative reactivity of the protein and of the free accepting groups.

Amino Acid Residues Modified in Aspartyl-tRNA Synthetase by Aspartylation. As could be anticipated, lysine residues are apparently the unique target of covalent aspartylation. However, it must be emphasized that the chemical differential labeling method does not detect a possible aspartylation of the free N-terminal group (Glx) of the enzyme subunit (Hounwanou et al., 1983): indeed, a difference of one Glx residue (out of 79) is well below the limit of experimental error in amino acid analysis. Similarly, weak ester or thioester bonds between aspartic acid and amino alcohol or amino thiol residues cannot be detected with the experimental approach used here.

The number of aspartylated lysine residues varies with the pH, from two residues at pH 6.0 to eight at pH 8.6. The number of labeled lysines does not significantly increase above the pH value (pH 9.6–11.5) whereas at pH 5.0 only one to two lysines are aspartylated.

Interestingly, the number of additional aspartic acid residues with α-NH_2 groups protected against dinitrophenylation decreases as the pH at which aspartylation was carried out increases: from about six residues per enzyme subunit at acidic or neutral pH to four or less at alkaline pH. These figures fit with the well-established difference in the p*K*s of α- and ϵ-NH_2 groups: aspartylation of aspartyl-tRNA synthetase at acidic pH will label 1 or 2 lysine residues each with a polyaspartyl moiety whereas at alkaline pH a greater number of lysines will be coupled to one or two aspartic acid residues. It is indeed quite striking to notice that the overall number of incorporated residues does not increase much between pH 6.0 and pH 8.6 (from 7 to 12). However, the formation of aspartic acid polymers on the synthetase at acidic pH is in apparent contradiction with the absence at these pHs of significant inhibition of protein aspartylation by free amino acids having α-NH_2 accepting groups (Table IV) since the free form of these amino acids accepts only poorly the activated amino acid; their acceptance ability appears only at alkaline pH (Table IV). This probably reflects different p*K*s of the α-NH_2 group of free or enzyme-linked amino acid.

At this stage, it must be stressed that the values listed in Table V do not reflect statistical labeling of lysine residues located at the surface of the protein; indeed, the number of tryptic aspartylated peptides is small (unpublished results), which means that the covalent modification occurs at a few specific sites. The possibility that all 40 lysine residues of each enzyme subunit are located at the surface of the protein, every one being equally accessible to the adenylate, looks unlikely given the conditions required to achieve their complete dinitrophenylation. Indeed, it was found essential to use both urea (8 M) and SDS (2% w/v) and long incubation periods with FDNB (24–48 h) for complete dinitrophenylation.

Structural and Catalytic Properties of Aspartylated Synthetase. As expected, the pH_i of the various forms of the synthetase shifts, after aspartylation, toward more acidic values, since ϵ-NH_2 lysyl groups (p*K* = 10.8) are substituted by γ-COOH aspartic acid groups (p*K* = 3.9). This pH_i shift can become sufficiently important to provoke the precipitation of the protein after extensive modification.

Isoelectric focusing of the aspartylated enzyme shows a great heterogeneity of the enzyme, which likely is related to the modification of the lysyl groups on the enzyme surface and to the incorporation at a given position of a variable number

of residues. However, a heterogeneity was already reported for the native enzyme. Since aspartylation is abolished when the synthetase is bound to its tRNA, the native enzyme should be essentially nonaspartylated, so that aspartylation cannot entirely account for this property.

SDS gel electrophoresis also shows a heterogeneity of the aspartylated enzyme since a doublet is observed. Qualitatively, this result is similar to that already reported for the native enzyme (Lorber et al., 1983a); here too the aspartylation process alone cannot account for this heterogeneity. It must be stressed that the increase in the molecular weight of the aspartylated subunits largely exceeds that expected from the number of residues incorporated (6000 for about 15 aspartic acid residues). The doublet observed on SDS gel electrophoresis for both the native and aspartylated enzymes and the high increase in the apparent molecular weight of the aspartylated enzyme can be related to their incomplete denaturation.

Gel filtration indicates an elongated shape for aspartyl-tRNA synthetase (apparent M_r 180 000; Lorber et al., 1983a). The same behavior is observed for the modified protein, indicating that aspartylation does not drastically affect the hydrodynamic volume of the enzyme.

The absence of significant modification of the rate constants of the synthetase for tRNA charging and ATP–PP_i exchange even after extensive aspartylation suggests that no amino acid group, able to accept the activated aspartic acid, is involved in the catalytic process. However, this is not the case for the substrate binding sites. The strong decrease in the affinity of $tRNA^{Asp}$ for the aspartylated synthetase indicates that modified lysyl groups are in the tRNA site. This could be anticipated since the contribution of ionic interactions involving lysyl groups of the protein in the association of tRNA has been reported for other systems (Baltzinger et al., 1979; Schulman et al., 1981). However, in contrast to that reported for phenylalanyl- and valyl-tRNA synthetases from bakers' yeast, aspartyl-tRNA synthetase has no lysyl group in the vicinity of the 3′-terminal adenosine tRNA binding site. The former systems are completely inactivated after covalent attachment by Schiff base formation of one periodate-oxidized tRNA per site (Baltzinger et al., 1979), whereas extensive aspartylation of the lysyl groups of aspartyl-tRNA synthetase does not affect the tRNA charging activity.

Lysyl groups have also been found in the amino acid activation site of bacterial aminoacyl-tRNA synthetases (Fayat et al., 1978) because the ATP–PP_i exchange activity was lost after covalent incorporation of one periodate-oxidized ATP per site. Since extensive aspartylation of aspartyl-tRNA synthetase does not affect ATP–PP_i exchange, no lysyl group seems to be present in the adenylation site. This is reinforced by the absence of significant modification of the affinity of aspartic acid and ATP for the aspartylated synthetase. Nevertheless, aspartylation modifies the binding properties of ATP on the dimeric synthetase. The biphasic double-reciprocal plot observed for the native enzyme indicates the existence of two nonequivalent binding sites differing in their affinity but not in their rate constant. Extensive aspartylation of the synthetase breaks the biphasicity of the Lineweaver and Burk plot, indicating a modification in the interrelation between the two ATP sites: both become equivalent for ATP binding with affinities slightly higher than that of the strong site in native enzyme and with unchanged rate constants. Finally, the absence in aspartyl-tRNA synthetase of essential lysyl groups is confirmed by enzyme labeling experiments with periodate-oxidized ATP and $tRNA^{Asp}$: both activities, ATP–PP_i exchange and tRNA charging, do not vary significantly after stoichiometric incorporation of one of these modified substrates per enzyme subunit (D. Kern et al., unpublished results).

It should be stressed that the mechanistic conclusion that could be drawn from this study resembles those derived from tRNA mischarging kinetics (Ebel et al., 1973). Indeed, in both cases a strict structural complementarity between tRNA and synthetase is not required to promote the aminoacylation of the tRNA: in mischarging experiments, the structures of tRNA vary for the same enzyme; here, a same tRNA interacts with different enzyme forms. Again, as already proposed (Ebel et al., 1973), this shows that specific synthetase–tRNA interactions do not alone govern tRNA aminoacylation and emphasizes the importance of the kinetic effects in the overall tRNA aminoacylation process.

Has the Aspartylation Process a Physiological or Catalytical Function? The question can be raised as to a possible physiological incidence of the aspartylation of aspartyl-tRNA synthetase. This process resembles that reported for beef pancreas tryptophanyl-tRNA synthetase (Kovaleva et al., 1978) but differs from it in several aspects. Only one tryptophanyl residue is incorporated per dimeric synthetase either in the absence of ATP or when tRNA is present, after tryptophanyl adenylate synthesis. Since tryptophan can directly be transferred to $tRNA^{Trp}$, the authors proposed a catalytic function for the tryptophanyl–enzyme intermediate and postulated its involvement in the overall $tRNA^{Trp}$ charging process. Obviously, aspartic acid covalently bound to the synthetase is not involved in tRNA charging.

A possible incidence of the aspartylation reaction could be an involvement in posttranscriptional modifications of cellular proteins. Although aspartyl adenylate remains bound to the enzyme in the presence of tRNA and thus is not able to react with potential protein acceptors and it has been proposed that under physiological conditions synthetases are essentially saturated by their cognate tRNA (Loftfield, 1972), this possibility cannot be rejected a priori. The reason is related with the high cellular concentration of elongation factors [e.g., Furano (1976)] that bind aminoacylated tRNA so that under certain conditions synthetases might be free from tRNAs.

Aspartylation as a Tool To Study Solution Structure of Proteins. Aspartylation of aspartyl-tRNA synthetase most likely occurs at accessible lysyl groups of the protein, some being located in the tRNA binding site. Because of the steric bulkiness of aspartyl adenylate, it can be assumed that the modified lysines are essentially located on the surface of the protein, a fact supported by the absence of enzyme inactivation even after extensive modification. Thus, the reaction can be used as a tool to map the surface topography of aspartyl-tRNA synthetase or of other proteins. Experiments are under way to explore this potentiality. For this approach, aspartyl-tRNA synthetase could be substituted by other aminoacyl-tRNA synthetases or systems able to generate acyl adenylate (thiokinases, luciferase) provided that conditions can be found where the acyl adenylate dissociates from enzyme. It might be argued that chemically synthesized acyl adenylates could be used as protein structural probes. The use of enzyme-generated acyl adenylate presents however a real advantage because the probe can be formed in adequate amounts inside the media where the structure of the protein will be mapped.

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Registry No. ATP, 56-65-5; Asp, 56-84-8; Tris, 77-86-1; aspartyl adenylate, 19046-78-7; aspartyl-tRNA synthetase, 9027-32-1; lysine, 56-87-1; alanine, 56-41-7; arginine, 74-79-3; asparagine, 70-47-3; cysteine, 52-90-4; glutamic acid, 56-86-0; glutamine, 56-85-9; methionine, 63-68-3; serine, 56-45-1; tyrosine, 60-18-4; spermine, 71-44-3; glutathione, 70-18-8; β-mercaptoethanol, 60-24-2.

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