Steck, T. L., and Fox, C. F. (1972), in Membrane Molecular Biology, Fox, C. F., and Keith, A. D., Ed., Stamford, Conn., Sinauer, p 27.
Steinhardt, J., and Reynolds, J. A. (1969), Multiple Equilibria in Proteins, New York, N.Y., Academic Press, p 239.
Tanford, C. (1968), *Adv. Protein Chem. 23,* 121.
Venyaminov, S Yu., Rajnavölgyi, É., Medgyesi, G. A., Gergely, J., and Závodszky, P. (1976), *Eur. J. Biochem. 67,* 81.
Victoria, E. J., Muchmore, E. A., Sudora, E. J., and Masouredis, S. P. (1975), *J. Clin. Invest. 56,* 292.
Virella, G., and Parkhouse, R. M. E. (1973), *Immunochemistry 10,* 213.
Wall, J. S. (1971), *Agric. Food Chem. 19,* 619.

Couplings between the Sites for Methionine and Adenosine 5′-Triphosphate in the Amino Acid Activation Reaction Catalyzed by Trypsin-Modified Methionyl-Transfer RNA Synthetase from *Escherichia coli*†

Guy Fayat, Michel Fromant, and Sylvain Blanquet*

ABSTRACT: Couplings (i.e., the changes in dissociation constant of one ligand upon ligation of another ligand) within the adenylating site of trypsin-modified methionyl-tRNA synthetase have been systematically analyzed with the use of analogues (or substructures) of methionine and ATP-Mg^{2+} molecules. Once bound to the enzyme, the specific amino acid side chain can couple as well with the binding of adenosine as with the binding of pyrophosphate. The primary coupling involving the subsites for adenosine and methioninol (an analogue of methionine lacking the carboxylate group) corresponds to a free energy of interaction, $\Delta G = 0.8$ kcal, which is enhanced by the carboxyl function of the amino acid or the α-phosphoryl group of 5′-adenosine nucleotides. Upon introduction of the carboxylate, the gain in Gibbs free energy of interaction for adenosine binding is equal to 0.3 kcal. Reciprocally the association of a 5′-adenosine nucleotide (ATP, ADP, or AMP) to the enzyme-bound methioninol is improved with respect to adenosine by values ranging from 1 to 2.2 kcal depending on the nucleotide considered and the presence of magnesium. These observations indicate that manifestation of major synergistic couplings requires neutralization of a cationic locus at the surface of the enzyme. Direct evidence that the carboxylate and α-phosphate groups are directed toward such a common enzymic locus is provided by the antagonistic couplings exerted on methionine binding either by ADP or AMP. Magnesium is involved in the couplings only when polyphosphoryl groups are engaged within the enzyme site. The effect of magnesium is to increase the free energies of couplings between ligands, one of which occupies the β and γ subsites of the ATP site. For instance, upon introduction of the divalent ion, the free-energy gain on methionine binding (in the presence of adenosine and pyrophosphate) rises up to 3.4 kcal. Thus, arising first of all from the specific binding of the amino acid is a series of self-amplifying coupled binding processes which take advantage of magnesium, the cofactor of catalysis. The resulting free energy which is no longer observed at the level of the methionine/ATP-Mg^{2+} coupling (prior to the reaction) is assumed to overcome the geometric and entropic requirements for aminoacyl adenylate synthesis.

Prior to their participation in polypeptide bond formation on ribosomes, tRNAs are specifically aminoacylated by aminoacyl-tRNA synthetases. The enzymatic aminoacylation of tRNA involves the activation of the amino acid through adenylate formation, followed by transfer of the aminoacyl moiety to a specific tRNA (for a review, see Kisselev and Favorova, 1974, and references therein). Several mechanisms have been proposed to account for the low rate of overall error in protein synthesis (Loftfield and Vanderjagt, 1972). The specificity of the aminoacylation reaction may be improved through "kinetical proofreading" (Hopfield, 1974; Ninio, 1975; Hopfield et al., 1976), "hydrolytic editing" (Fersht and Kaethner, 1976), or "chemical proofreading" (von der Haar and Cramer, 1976) of misacylated tRNAs on the synthetase itself. Such mechanisms may be of the utmost importance in order to discriminate against misactivation of amino acids which are isosteric with the correct one. While several examples of misactivation of amino acids by aminoacyl-tRNA synthetases have been discovered and are well documented (Baldwin and Berg, 1966), it is nevertheless clear that in most cases activation of the correct amino acid remains a key feature of the overall specificity of the aminoacylation reaction.

The coupling between ligands (i.e., the change in the dissociation constant of one ligand upon binding of another ligand) which has been observed within the adenylating site of several aminoacyl-tRNA synthetases (Kosakowski and Holler, 1973; Fayat and Waller, 1974; Blanquet et al., 1975b; Holler et al., 1975) may well be an important factor contributing to the specificity of the amino acid activation reaction.

† From the Laboratoire de Biochimie-Ecole Polytechnique, Laboratoire associé No. 240 du Centre National de la Recherche Scientifique, 91120-Palaiseau, France. *Received June 23, 1976.* This study was supported in part by the Commissariat à l'Energie Atomique (Saclay, France) and by the Délégation Générale à la Recherche Scientifique et Technique.

In the case of methionyl-tRNA synthetase (L-methionine; $tRNA^{Met}$ ligase (AMP-forming), EC 6.1.1.10), methionine and adenosine molecules are synergistically coupled on the enzyme independently of the presence of magnesium. The resulting ternary complex is markedly stabilized by pyrophosphate. However, this latter synergistic coupling requires magnesium (Blanquet et al., 1975a,b). Contrary to adenosine, ATP-Mg^{2+} is not coupled to the binding of methionine. This point has been recently demonstrated by stopped-flow analysis of the reversible adenylation reaction (Hyafil et al., 1976). It was therefore suggested that all of the coupling binding energy between methionine and the adenosine and pyrophosphate moieties of ATP-Mg^{2+} was consumed to overcome the electrostatic repulsion between the reacting phosphoryl and carboxyl groups within the active center of the synthetase. In this work, analogues (or substructures) of the methionine and ATP-Mg^{2+} molecules will be systematically analyzed for their contribution to the coupling process within the single site of trypsin-modified methionyl-tRNA synthetase (Cassio and Waller, 1971; Blanquet et al., 1974, 1975b; Hyafil et al., 1976).

We will conclude that coupling requires the binding of methionine to its specific enzyme. Thus, ensuing from the specificity of amino acid binding is a free energy of coupling which is then utilized to provide the driving force for catalysis (i.e., to overcome the geometric and entropy requirements for aminoacyl adenylate synthesis).

Materials and Methods

Homogeneous trypsin-modified methionyl-tRNA synthetase is derived from the native enzyme of the *Escherichia coli* strain EM20031 carrying the F32 episome as described previously (Cassio and Waller, 1971). Enzyme concentration is determined from its absorbancy at 280 nm as described elsewhere (Blanquet et al., 1973).

Adenosine, ATP (Na), and 5′-AMP (Na) salts were purchased from Boehringer (Mannheim). 5′-ADP was from Sigma. L-Methionine, PP (Na), and imidazole were from Merck. L-Methioninol was a gift from Dr. M. Robert-Gero or purchased from Fluka. 5′-Carboxyladenosine was synthesized by Sefochem and 3-methylthiopropylamine was from Eastman Kodak. [^{14}C]Adenosine, 5′-[^{14}C]AMP, and 5′-[^{14}C]ATP were obtained from Amersham with respective specific radioactivities of 390, 500, and 500 mCi/mmol. [^{32}P]PP[1] (Na) (100 mCi/mmol) was delivered by The Commisariat à l'Energie Atomique. The purity of the labeled nucleosides and nucleotides was assayed by thin-layer chromatography on cellulose, using the solvent system isobutyric acid/water/ammonia (66:33:1, v/v). ADP was purified by paper chromatography using the same solvent system. Concentrations of methionine and methioninol were determined by titration with ninhydrin while their sulfoxide contents were evaluated by chromatography on cellulose with the solvent system 2-propanol/water (7:3, v/v). The p*K*s of methionine, methioninol, ATP, ADP, AMP, and pyrophosphate were determined in water at 25 °C under nitrogen using the pHM64 pH meter from Radiometer with the TTT60 Titrator accessory. In these experiments, the methionine and methioninol solutions (10 mM) contained 50 mM KCl. The ATP, ADP, and AMP (disodium salts) solutions (10 mM) were prepared with or without 20 mM $MgCl_2$. The pyrophosphate (tetrasodium salt) solution (5 mM) was prepared with or without 7 mM $MgCl_2$.

Isotopic [^{32}P]PP-ATP exchange was assayed as previously described (Blanquet et al., 1974, 1975a) in standard buffer containing 20 mM imidazole-HCl at pH 7.6 (25 °C), 0.1 mM EDTA (acid form), and 10 mM 2-mercaptoethanol. The magnesium content was as indicated in the Results section. The initial rate of exchange was measured in the presence of 1 mM methionine plus methioninol concentrations varying from 0 to 2 mM. The ATP concentration was varied from 0.1 to 1 mM in the presence of pyrophosphate concentrations varying from 0.63 to 2.07 mM. The data were analyzed on a Wang 2200B computer using a multilinear regression program. Equilibrium dialyses were performed in standard buffer at 25 °C as previously described (Blanquet et al., 1975b) in the presence of the magnesium concentrations indicated in Table IV. The corresponding dissociation constants were determined graphically with an error estimated from the dispersion of the experimental values when plotted according to Scatchard. In cases where the dissociation constants were significantly larger than the enzyme concentrations used, the plots were obtained assuming a unique binding site on the trypsin-modified enzyme (Blanquet et al., 1975b).

Protein fluorescence was measured at 25 °C with a spectrofluorimeter described elsewhere (Hyafil et al., 1976). The fluorescence was excited at 295 nm and followed at 332 nm. The method of titration was as previously described (Blanquet et al., 1975b). Dissociation constants, K_d, were obtained by regression of the linear function $\phi = \phi_\infty - K_d(\phi - \phi_0)/[AA]_0$ where $[AA]_0$ represents the total methionine or methioninol concentration in the course of titration and is equivalent to free methionine or methioninol since the concentration of modified enzyme (0.3 μM) was negligible compared to the K_d values obtained. ϕ_0 and ϕ, respectively, indicate the relative fluorescence of the enzyme before and in the course of titration by methionine or methioninol. ϕ_∞ corresponds to the relative fluorescence of the enzyme at the end of the titration. Further analysis of the K_d values as a function of coupled ligands was achieved with multiparameters iterative nonlinear regression procedures on the Wang 2200B computer.

Results

(A) *Effect of ATP and Related Compounds on the Binding of L-Methioninol, an Analogue of Methionine Lacking the Carboxyl Group*

(1) *Inhibition by L-Methioninol of the Isotopic ATP-[^{32}P]PP Exchange Reaction Catalyzed by Trypsin-Modified Methionyl-tRNA Synthetase.* The general rate equation describing the initial velocity of exchange as a function of the substrate concentrations has been elucidated and extended to the case where competitive inhibitors of ATP (adenosine and analogues) are present in solution (Blanquet et al., 1974, 1975a). In the present case, L-methioninol behaves as a competitive inhibitor of methionine in the ATP–PP exchange assay (Cassio et al., 1967; Blanquet et al., 1972). Consequently, the following complexes of methioninol with the enzyme may be assumed: E·AAol, E·AAol·PP, and E·AAol·ATP, with E and AAol representing enzyme and L-methioninol, respectively. Using the formalism previously described (Blanquet et al., 1974), the expression for $[E]_t/v$, the inverse of the initial velocity of exchange, is a linear function of [AAol], the inhibitor

[1] Abbreviations used are: E, AA, Ado, and PP, enzyme, L-methionine, adenosine, and pyrophosphate, respectively; AAol and MTPA, L-methioninol and 3-methylthiopropylamine, respectively. The equilibrium constants are symbolized by two indices. The lower indicates the ligand in equilibrium with methionyl-tRNA synthetase saturated by the ligands represented in the upper index.

TABLE I: Equilibrium Constants for Methioninol on Trypsin-Modified Methionyl-tRNA Synthetase as Derived from Analysis of the Isotopic [^{32}P]PP-ATP Exchange Reaction.[a]

$[MgCl_2]$ (mM)	K_{AAol} (μM)	$K_{AAol\cdot ATP}$ (M)^2	$K_{ATP}^{AAol} = K_{AAol\cdot ATP}/K_{AAol}$ (μM)	$K_{AAol}^{ATP} = K_{AAol\cdot ATP}/K_{ATP}$ (μM)
7		$(3.8 \pm 0.1) \times 10^{-9}$		
20	446 ± 99	$(7.8 \pm 0.5) \times 10^{-9}$	17.5	6.5

[a] Initial rates of the methionine-dependent ATP-PP isotopic exchange reaction are measured at 25 °C in standard buffer (pH 7.6) with the magnesium concentrations indicated in the table. Permutation of the methioninol, ATP, and PP concentrations within the exchange assay enables determination of the parameters which are presented in the table (Blanquet et al., 1975a). The equilibrium symbols are defined as follows: $K_{AAol} = [E][AAol]/[E\cdot AAol]$, $K_{ATP} = [E][ATP]/[E\cdot ATP]$, $K_{AAol\cdot ATP} = [E][AAol][ATP]/[E\cdot AAol\cdot ATP]$, $K_{ATP}^{AAol} = [E\cdot AAol][ATP]/[E\cdot AAol\cdot ATP]$, and $K_{AAol}^{ATP} = [E\cdot ATP][AAol]/[E\cdot AAol\cdot ATP]$. The latter constant is calculated in the table by using the value $K_{ATP} = 1.2$ mM determined elsewhere (Blanquet et al., 1974).

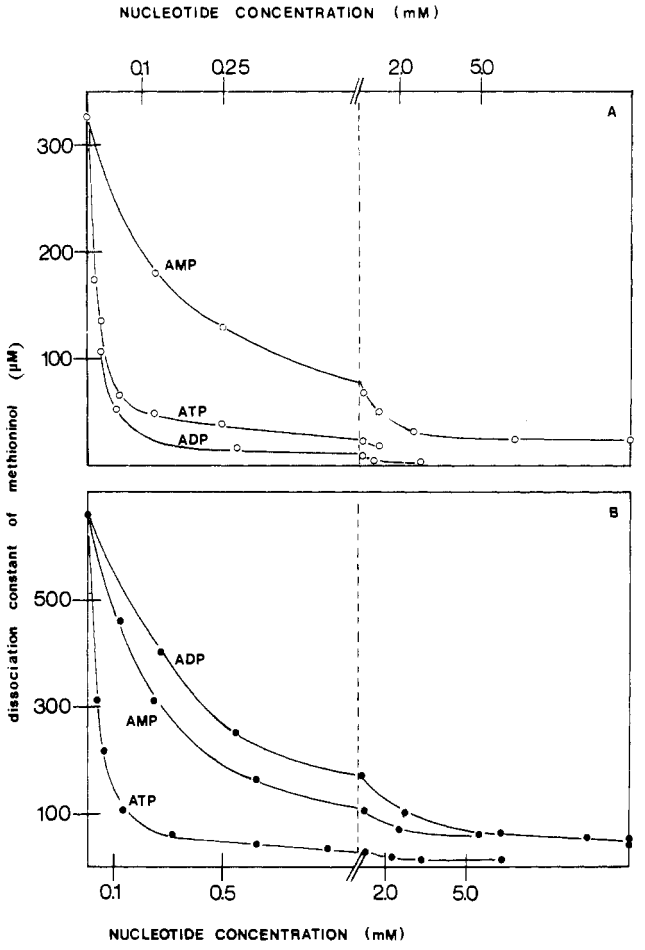

FIGURE 1: Effect of magnesium chloride on the dissociation constants of methionine (●) or methioninol (○) from trypsin-modified methionyl-tRNA synthetase at 25 °C in standard buffer (7.6).

concentration (Blanquet et al., 1975a), and the slope of $[E]_t/v$ with respect to [AAol] is given by:

$$\frac{d([E]_t/v)}{d[AAol]} = \frac{K_{eq}}{k_{app}[AA]} \times \left[\frac{1}{K_{AAol\cdot ATP}} + \frac{1}{[ATP]}\left(\frac{1}{K_{AAol}} + \frac{[PP]}{K_{AAol\cdot PP}}\right)\right] \quad (1)$$

with $K_{AAol} = [E][AAol]/[E\cdot AAol]$, $K_{AAol\cdot PP} = [E][AAol][PP]/[E\cdot AAol\cdot PP]$ and $K_{AAol\cdot ATP} = [E][AAol][ATP]/[E\cdot AAol\cdot ATP]$.

The equilibrium constants of the above relation can be resolved by measuring the slopes $d([E]_t/v)/d[AAol]$ for different permutations of ATP and PP concentrations while keeping the methionine concentration constant in all experiments. The slope is found to be independent of the pyrophosphate concentration in the millimolar range, showing that there is no coupling of pyrophosphate with the enzyme-bound methioninol. Moreover, the slope is not very dependent on the ATP concentration in the solution, showing that the role of the E·AAol complex is small in the inhibition described by eq 1. The $(K_{AAol\cdot ATP})^{-1}$ term is predominant in eq 1. This means that the E·AAol·ATP complex is the main factor responsible for the inhibition (Table I). The experiments have been performed in the presence of 7 mM or 20 mM $MgCl_2$. As reflected in Table I increasing the divalent ion concentration depressed the extent of methioninol inhibition. This result should be related to the observation in the following section that increasing the divalent ion concentration enhances the dissociation constant of methioninol. The calculated equilibrium constants of Table I indicate a large coupling betweeen AAol and ATP-Mg^{2+}, in agreement with a previous observation obtained by equilibrium dialysis that methioninol had a marked cooperative effect on the binding of ATP-Mg^{2+} (Fayat and Waller, 1974).

(2) *Coupling of Methioninol and ATP or Related Compounds as Studied by Fluorescence at Equilibrium.* As described earlier (Blanquet et al., 1972), methioninol enhances the tryptophan fluorescence of methionyl-tRNA synthetase as does methionine. The resulting saturation hyperbola allows the determination of a methioninol dissociation constant which is a function of the coupled ligands. In the simplest case the apparent dissociation constant for methioninol is ruled by the following relationship:

$$K_d = K_{AAol}(1 + K_{ATP}/[ATP])/(1 + K_{ATP}^{AAol}/[ATP])$$

with $K_{ATP} = [E][ATP]/[E\cdot ATP]$ and $K_{ATP}^{AAol} = [E\cdot AAol][ATP]/[E\cdot AAol\cdot ATP]$ where ATP has been taken as an example of a ligand coupled to methioninol. The K_d values collected for different concentrations of the coupled ligand were fitted to the above relationship with the help of a three-parameter iterative nonlinear regression program.

(a) ATP, ADP. Figure 1 illustrates the variations of the dissociation constant of L-methioninol with the trypsin-modified methionyl-tRNA synthetase as a function of the ATP or ADP concentrations. The constant is dramatically decreased on addition of these nucleotides both in the absence and in the presence of the divalent magnesium ion. The dissociation constants for couplings were determined as indicated above and are presented in Table II. The data reveal that the divalent ion decreases the affinity of methioninol for the free enzyme.

This phenomenon is illustrated by Figure 2 where K_{AAol} is

TABLE II: Binding of Methioninol to Trypsin-Modified Methionyl-tRNA Synthetase. The Effects of Bound ATP, ADP, AMP, or Adenosine.[a]

X =	[$MgCl_2$] (mM)	K_{AAol} (μM)	K_X (μM)	K_X^{AAol} (μM)	$K_{AAol}^X = K_{AAol}K_X^{AAol}/K_X$ (μM)	$C_X = K_{AAol}/K_{AAol}^X$
ATP	0	330 ± 3	250 ± 60	13.5 ± 1.0	17.8	18.5
ATP-Mg^{2+}	20	662 ± 9	1 854 ± 739	30 ± 1.5	10.7	62
ADP	0	330 ± 15	1 950 ± 1282	13 ± 0.6	2.2	150
ADP-Mg^{2+}	20	706 ± 14	7 890 ± 3449	306 ± 26	27.4	26
AMP	0	330 ± 3	2 417 ± 279	134 ± 5	18.3	18
AMP-Mg^{2+}	20	662 ± 9	3 951 ± 1605	202 ± 24	33.8	19.5
Ado	0	335 ± 16	14 400 ± 9900	3700 ± 1500	86	3.9
Ado-Mg^{2+}	7	459 ± 13	23 100 ± 22500	6100 ± 3000	121	3.8

[a] The binding constant of methioninol (k_d) to the enzyme was measured by fluorescence at 25 °C in standard buffer (pH 7.6) in the presence of various concentrations of the nucleoside or nucleotide under study (X). The relationship $K_d = K_{AAol}(1 + (X)/K_X)/(1 + (X)/K_X^{AAol})$ was fitted to the experimental values of Figures 1 and 3 (panel A). Values of the corresponding parameters are given in the table with their standard errors. C_X is the coupling exerted by the nucleotide or nucleoside X on methioninol binding parameters, i.e. the ratio of the binding constants of methioninol to enzyme at zero and infinite concentration of the nucleotide, X.

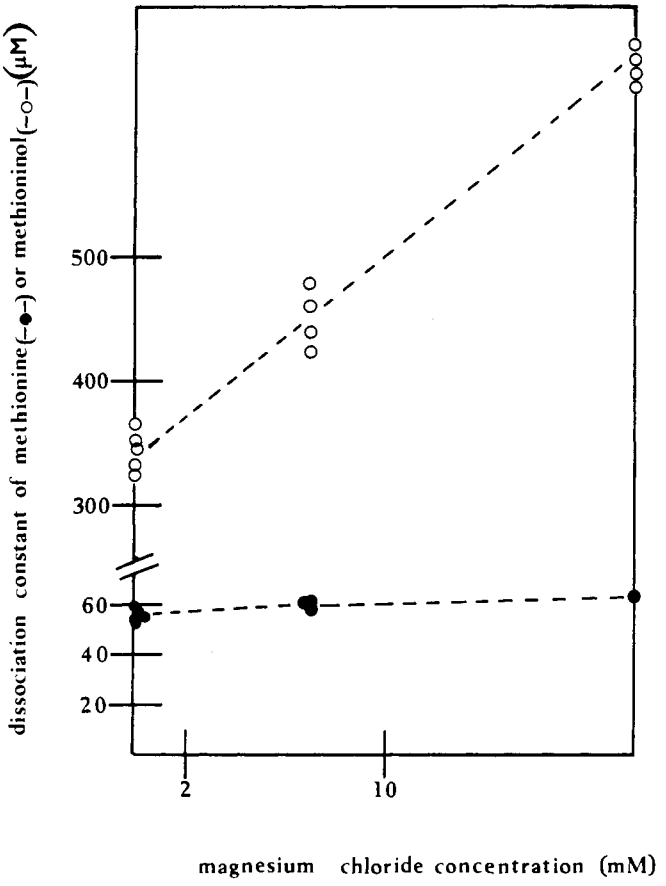

FIGURE 2: Dissociation constant of methioninol from trypsin-modified methionyl-tRNA synthetase as a function of synergistic nucleotides. The enzyme (0.3 μM) is titrated by methioninol in the presence of various amounts of 5′-ATP, 5′-ADP, and 5′-AMP in the absence (panel A) and in the presence (panel B) of a 20 mM free magnesium concentration at 25 °C, in standard buffer (pH 7.6). Each methioninol binding constant is obtained by varying methioninol from zero to total saturation of the enzyme. The continuous lines correspond to the variation of the dissociation constant calculated from the values of Table II.

plotted for different magnesium concentrations. The divalent ion appears to compete with methioninol whereas methionine binding is not much affected under the same conditions, as reported earlier (Blanquet et al., 1972; Fayat and Waller, 1974). Since the amino groups of methionine and methioninol are protonated under our standard conditions (respective pKs of 9.2 and 8.9), the difference is attributed only to the presence of the carboxylate subgroup of the amino acid. In the cases of other activating enzymes (Holler et al., 1975) a similar effect has been interpreted as reflecting electrostatic repulsion by the cation binding close to the active site. The repulsion would be compensated through attraction by the carboxylate in the case of the intact amino acid. However that may be, this ion effect is not involved in the development of the couplings which are the object of the investigation.

The values of Table II show that ATP binding is sensitive to the presence of the divalent ion. As reported earlier, the metal-ATP complex is a weaker ligand of the free enzyme than the free ATP molecule (Fayat and Waller, 1974). However, the binding of ATP-Mg^{2+} is strongly enhanced by L-methioninol.

The values of Table II indicate that the magnesium-ADP complex also is a weaker ligand than the free ADP molecule. Indeed, the free ADP molecule behaves on the free enzyme as the magnesium-ATP complex, while ADP-Mg^{2+} resembles AMP or adenosine. On the other hand, in contrast to the case of ATP, the coupling of L-methioninol to ADP is stronger than to ADP-Mg^{2+}.

(b) AMP. AMP also is coupled with methioninol. The dissociation constant of methioninol is vastly decreased upon addition of the nucleotide (Figure 1 and Table II). The binding parameters of 5′-AMP are slightly affected by the presence of magnesium. It may be noted that under our conditions (20 mM total magnesium) the ratio of magnesium-bound AMP is significant (about 50% from Belaich and Sari (1969)). Our result tends to indicate that the enzyme selects the free AMP species for binding. Moreover, the coupling of 5′-AMP on the binding of methioninol, when calculated as the ratio K_{AAol}/K_{AAol}^{AMP}, is not sensitive to the divalent ion.

(c) Adenosine. As in the case of methionine (Blanquet et al., 1975b), the coupling between methioninol and the nucleoside is insensitive to magnesium (Figure 3, panel A). In the presence or absence of this metal, saturating amounts of adenosine decrease the dissociation constant of methioninol roughly by a factor of four, while the corresponding constant of methionine was decreased by about six times (Table II).

(d) Pyrophosphate. In the absence as well as in the presence

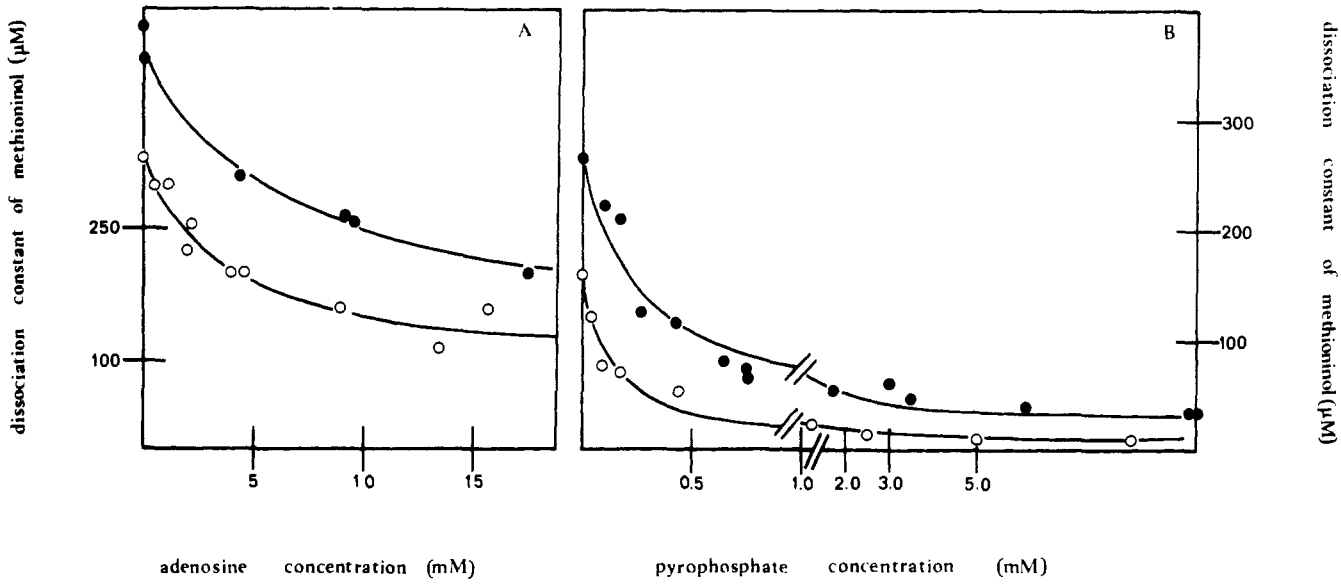

FIGURE 3: Dissociation constant of methioninol from trypsin-modified methionyl-tRNA synthetase as a function of the synergistic combination of adenosine plus pyrophosphate. The enzyme (0.3 μM) is titrated by methioninol at 25 °C in standard buffer (pH 7.6) in the presence of various amounts of adenosine (panel A), in the presence (●) or absence (○) of 7 mM free magnesium. In panel B, the enzyme is titrated by methioninol in the presence of various amounts of pyrophosphate and of a fixed adenosine concentration (9 mM), with (●) or without (○) 7 mM free magnesium. Calculations of free magnesium concentrations assume that one molecule of adenosine or pyrophosphate binds, respectively, zero and one molecule of divalent magnesium ion. The continuous lines correspond to the variation of the dissociation constant calculated from the values of Tables II and III.

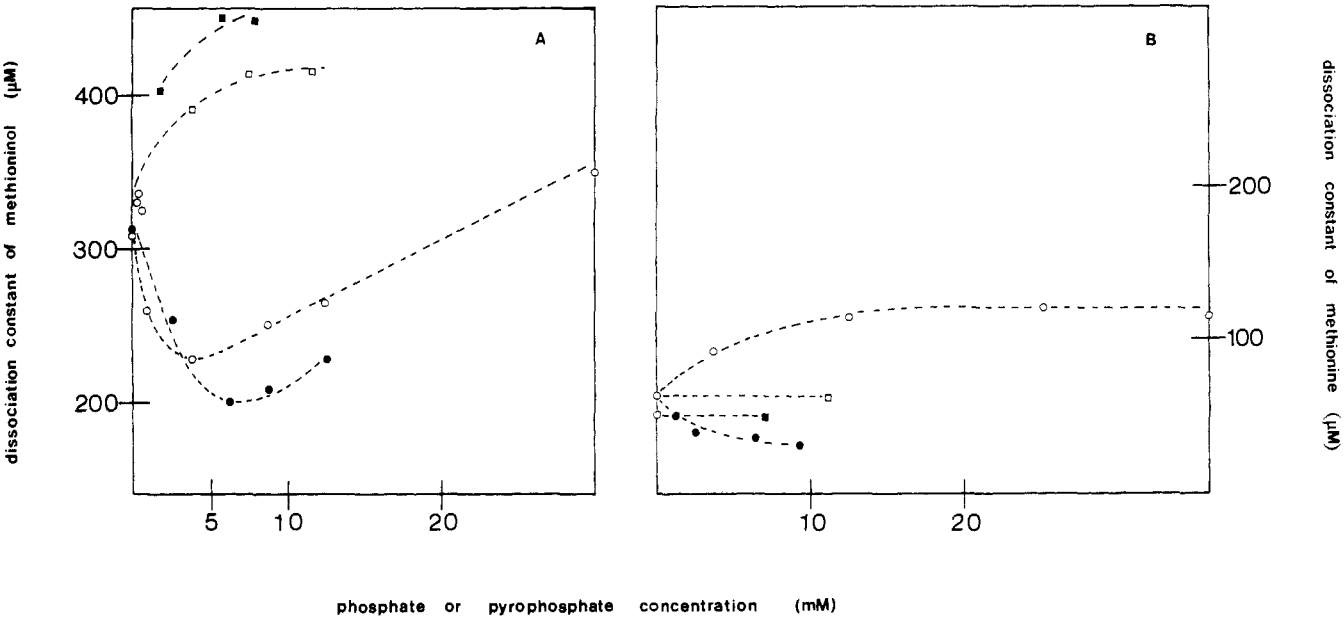

FIGURE 4: Effects of pyrophosphate and phosphate on the dissociation constants of methioninol (panel A) or methionine (panel B) from trypsin-modified methionyl-tRNA synthetase. The enzyme (0.3 μM) is titrated by the amino acid or its alcohol derivative at 25 °C in standard buffer (pH 7.6) in the presence of various amounts of phosphate (□, ■) or pyrophosphate (○, ●). The experiments are monitored in the presence (●, ■) or absence (○, □) of magnesium. The presence of magnesium in these experiments was ensured by stoichiometric amounts of magnesium and pyrophosphate or phosphate.

of magnesium, pyrophosphate is weakly coupled to methioninol binding (Figure 4, panel A). This effect may be due to the binding of pyrophosphate at the site for the ATP phosphate groups. However, at higher concentration of pyrophosphate, the coupling becomes antagonistic. This could correspond to the binding of a second pyrophosphate molecule within the phosphate sites, resulting in electrostatic and steric hindrances between the bound pyrophosphate molecules. Alternatively, the inhibition could reflect ionic effects since adding phosphate has the same consequence (Figure 4).

(e) Adenosine plus Pyrophosphate. Since coupling on the enzyme-adenosine complex between methionine and pyrophosphate had been recently reported which was dependent on the presence of the divalent ion (Blanquet et al., 1975b), it was of interest to investigate the case of methioninol. It is clearly shown in Figure 3 (panel B) that methioninol and pyrophosphate binding is also strongly coupled on the enzyme-nucleoside complex. However, in contrast to methionine, this coupling, which now involves an alcohol function instead of a carboxylic residue, does not require magnesium. The data of

TABLE III: Binding of Methioninol to Trypsin-Modified Methionyl-tRNA Synthetase. The Effects of Bound Pyrophosphate/Adenosine and Pyrophosphate/5′-AMP.[a]

X =	[$MgCl_2$] (mM)	K_{AAol}^{X} (μM)	K_{PP}^{X} (μM)	$K_{PP}^{X\cdot AAol}$ (μM)	$K_{AAol}^{X\cdot PP}$ (μM)	$C_X = K_{AAol}/K_{AAol}^{X\cdot PP}$
Ado	0	100 ± 6	700 ± 500	89 ± 23	13	25.4
Ado-Mg^{2+}	(PP) + 7	176 ± 11	1 140 ± 600	185 ± 43	28.5	16.1
AMP-Mg^{2+}	(PP)	22 ± 1	11 000 ± 7000	2100 ± 600	4.2	78

[a] In the case of adenosine, the presence of pyrophosphate–magnesium on the enzyme was ensured with a 7 mM excess of metal ion with respect to the PP concentration. In the case of 5′-AMP the presence of pyrophosphate–magnesium is ensured by stoichiometric amounts of metal and pyrophosphate. Precipitation occurs if more metal is added. Values in the table with their standard errors were obtained by fitting the data represented in Figure 3 (panel B) and Figure 5. These data have been respectively fitted to the relationship $K_d = K_{AAol}^{Ado}(1 + K_{Ado}/[Ado] + [PP]/K_{PP}^{Ado})/(1 + K_{Ado}^{AAol}/[Ado] + [PP]/K_{PP}^{Ado\cdot AAol})$ (where the fact that adenosine (Ado) was not saturating in the experiments performed has been taken into account), and to the relationship $K_d = K_{AAol}^{AMP}(1 + [PP]/K_{PP}^{AMP})/(1 + [PP]/K_{PP}^{AMP\cdot AAol})$.

Figure 3 have been fitted to the following relationship:

$$K_d = K_{AAol}^{Ado}(1 + K_{Ado}/[Ado] + [PP]/K_{PP}^{Ado})/(1 + K_{Ado}^{AAol}/[Ado] + [PP]/K_{PP}^{Ado\cdot AAol})$$

where the fact that adenosine (Ado) was not saturating in the experiments has been taken into account. The thermodynamic parameters are defined as follows: $K_{Ado} = [E][Ado]/[E\cdot Ado]$, $K_{Ado}^{AAol} = [E\cdot AAol][Ado]/[E\cdot AAol\cdot Ado]$, $K_{AAol}^{Ado} = [E\cdot Ado][AAol]/[E\cdot AAol\cdot Ado]$, $K_{PP}^{Ado} = [E\cdot Ado][PP]/[E\cdot Ado\cdot PP]$, and $K_{PP}^{Ado\cdot AAol} = [E\cdot AAol\cdot Ado][PP]/[E\cdot AAol\cdot Ado\cdot PP]$. Their calculated values are given in Table III.

(f) AMP plus Pyrophosphate. This combination simulates the ATP molecule but it has one more oxygen atom and two more negative charges at the sites for the α and β phosphates. Pyrophosphate reduces the coupling of AMP and methioninol as shown in Figure 5 (panel A). It must be noted that this effect occurs at considerable concentrations of pyrophosphate.

In the presence of magnesium, the behavior of pyrophosphate is completely different. It is synergistically coupled with methioninol on the enzyme–AMP complex (Figure 5B, Table III) and provides an extent of coupling for methioninol binding which matches the excellent coupling provided by the ATP–Mg^{2+} molecule. Lastly, the specificity of pyrophosphate on the methioninol–AMP coupling was verified with the use of phosphate (Figure 5).

(3) *Coupling of Methioninol and ATP or Related Compounds as Studied by Dialysis at Equilibrium.* The series of dialysis experiments which are summarized in Table IV provides independent evidence for the different couplings found in the previous section. The values obtained for the various equilibrium constants are in satisfying agreement with the corresponding ones derived from the fluorescence data. This provides a check on the validity of the above analysis.

(B) *Effect of ATP and Related Compounds on the Binding of L-Methionine.*

(a) *ATP.* It has been shown elsewhere (Hyafil et al., 1976) that methionine and ATP–Mg^{2+} react without coupling to form the reactive E·AA·ATP–Mg^{2+} ternary complex. Therefore, introduction of the crucial α-phosphate into the catalytic site of methionyl-tRNA synthetase obliterates the major synergistic coupling exhibited between methionine and the adenosine and PP–Mg^{2+} parts of the ATP–Mg^{2+} molecule (Blanquet et al., 1975b). Alternatively, introduction of the carboxylate of methionine obliterates the coupling exerted between methioninol and ATP–Mg^{2+}.

(b) *ADP and AMP.* Equilibrium constants of methionine with the enzyme, in the presence of various concentrations of these nucleotides, have been derived from fluorescence titrations (Blanquet et al., 1974, 1975b). These constants are altered by increasing the concentrations of 5′-ADP or 5′-AMP in the solution. Their variations, summarized in Figure 6, exhibit antagonistic coupling between methionine and the nucleotides.

While magnesium has no important effect on the antagonistic coupling in the case of AMP, the divalent ion affects the behavior of ADP. This result probably reflects the respective effects of magnesium on the binding of the nucleotides to the free enzyme (Table II). Indeed, the affinity of ADP for the enzyme is much more decreased by magnesium than that of AMP. In the case of AMP, fitting our experimental results enables us to calculate coupling factors defined as K_{AA}/K_{AA}^{AMP}, respectively, equal to 0.14 and 0.2 in the absence and in the presence of 7 mM $MgCl_2$.

(c) *Pyrophosphate.* Since pyrophosphate has been shown in Figure 4 to exhibit synergistic coupling on methioninol binding, we have examined the effect of PP on methionine binding to the enzyme. The results of panel B in Figure 4 reveal that, as in the case of the amino alcohol, the pyrophosphate–magnesium decreases the K_{AA} value by about twofold. However, in the absence of magnesium, pyrophosphate couples antagonistically to methionine. This marked difference with the case of methioninol is accounted for by the presence of the negatively charged carboxyl group of methionine. Specificity for these effects was checked with the use of phosphate.

(d) *AMP plus Pyrophosphate.* Finally we have measured the effect of pyrophosphate on the antagonistic coupling between methionine and AMP. As shown in Figure 6 (panel C), pyrophosphate alone destabilizes weakly the ternary enzyme–methionine–AMP complex. In contrast, pyrophosphate–magnesium stabilizes this complex. It decreases by about two times the K_{AA}^{AMP} value, as it does in the absence of AMp. Thus, it appears that, as in the case of the methionine plus adenosine combination, pyrophosphate requires magnesium to act synergistically.

(e) *Miscellaneous.* It has been demonstrated elsewhere that 3-methylthiopropylamine, an analogue of methionine lacking the carboxyl group, exerted a cooperative effect on the binding of ATP–Mg^{2+} to the trypsin-modified methionyl-tRNA synthetase (Fayat and Waller, 1974). With the use of protein fluorescence (Blanquet et al., 1972), we have now verified that this methionine analogue couples like methioninol, with the difference that their respective equilibrium constants with the free enzyme, K_{MTPA} and K_{AAol}, are respectively equal to 1200 and 330 μM. In parallel, we have examined the properties of 5′-carboxyladenosine, an analogue of adenosine, the negative carboxyl charge of which could simulate the α-phosphoryl

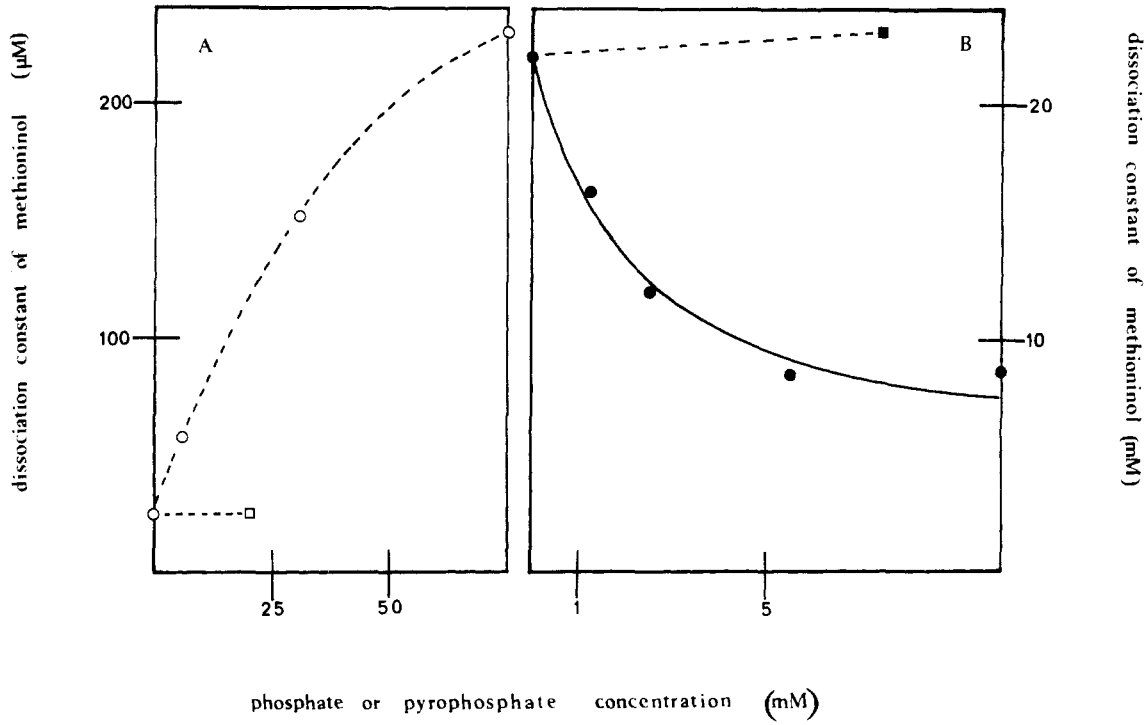

FIGURE 5: Dissociation constant of methioninol from trypsin-modified methionyl-tRNA synthetase as a function of the combination of 5′-AMP plus pyrophosphate of phosphate. The enzyme (0.3 μM) is titrated by methioninol at 25 °C in standard buffer (pH 7.6) in the presence of various amounts of pyrophosphate (○, ●) or phosphate (□, ■) and of a fixed concentration of 5′-AMP (12 mM). The experiments are monitored in the presence (●, ■) or absence (○, □) of magnesium chloride. The presence of magnesium was ensured by stoichiometric amounts of magnesium and pyrophosphate or phosphate. The continuous line in panel B represents the variation of the dissociation constant calculated from the values of Table III.

TABLE IV: Equilibrium Constants of Ligands Studied by Equilibrium Dialysis with Trypsin-Modified Methionyl-Transfer RNA Synthetase in the Absence or Presence of Synergistic Protagonists. Confirmation of the Results Obtained by Fluorescence.[a]

		Synergistic protagonists					
Ligand under study	[KCL] (mM)	[L-Methioninol] (mM)	[Adenosine] (mM)	[Pyrophosphate] (mM)	[Magnesium] (mM)	[Enzyme] μM)	Equilibrium constants (μM)
ATP	100					210	180 ± 20
ATP	100	20				50	27 ± 5
ATP	100				20	200	2000 ± 1000
ATP		20			7	50	53 ± 10
AMP	100					100	>3000
AMP	100	20				100	250 ± 20
AMP	100	20		2		100	290 ± 40
AMP					7	100	>3000
AMP		20			7	100	260 ± 30
AMP		20		2	7	100	160 ± 40
Ado	100	20				97	2000 ± 600
Ado	100	20		2		97	480 ± 50
Ado		20			7	97	1900 ± 500
Ado		20		2	7	97	1076 ± 400
PP	20	1.5	18			40	90 ± 10

[a] The equilibrium constants correspond to the dissociation constant of the ligand under study in the presence of the indicated synergistic protagonists. These constants were obtained at 25 °C in standard buffer (pH 7.6) in the presence of the KCl and $MgCl_2$ concentrations indicated in the table. The errors presented in this table were estimated from the dispersion of the Scatchard plot.

charge of 5′-AMP toward the catalytic center. When measured by fluorescence, in the presence of a 20 mM concentration of 5′-carboxyladenosine, the dissociation constant of methionine is increased by 2.3 times while the dissociation constant of methioninol is lowered by 2 times. It was then verified that, as in the case of AMP, pyrophosphate reduces the coupling between methioninol and 5′-carboxyladenosine.

Finally, the important role of the side chain of methionine in the processes which are described in this article was emphasized with the use of glycine. Even in the presence of a major synergistic combination such as adenosine plus PP-Mg^{2+}, glycine at a concentration of 7 mM does not bind to the enzyme nor does it affect the binding of methioninol according to the fluorescence assay.

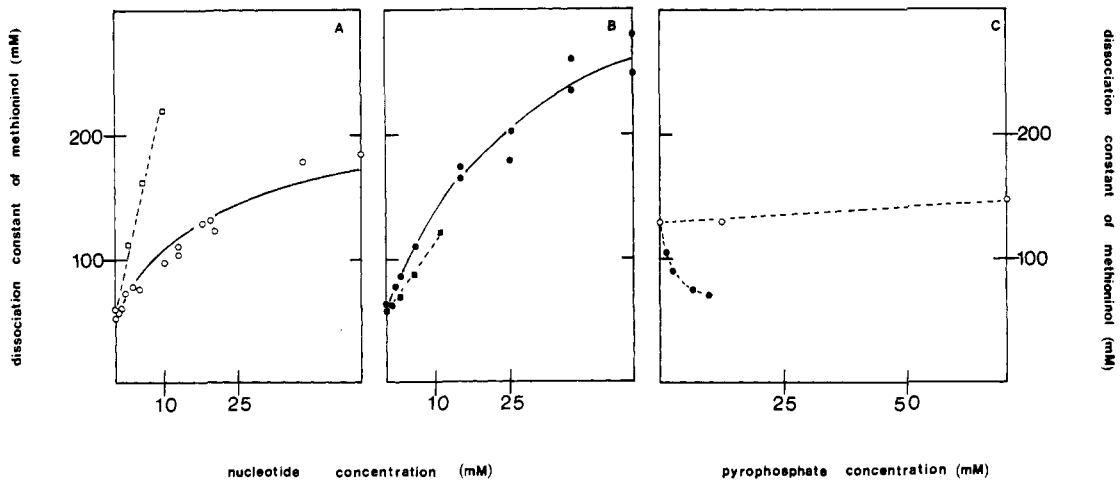

FIGURE 6: Dissociation constant of methionine from trypsin-modified methionyl-tRNA synthetase as a function of the antagonistic ligands 5′-AMP and 5′-ADP. In panels A and B the enzyme (0.3 μM) is titrated by methionine at 25 °C in standard buffer (pH 7.6), in the presence of various amounts of 5′-AMP (○, ●) or 5′-ADP (□, ■) without (panel A) or with (panel B) 7 and 20 mM free magnesium concentrations, respectively, in the cases of AMP and ADP. In panel C, the enzyme is titrated by methionine in the presence of various amounts of pyrophosphate and of a constant concentration of 5′-AMP (20 mM) with (●) and without (○) magnesium chloride. In this case the presence of magnesium was ensured by stoichiometric amounts of magnesium and pyrophosphate. The continuous lines plotted in this figure correspond to the variation of the dissociation constant calculated from the coupling values which are indicated under Results.

Conclusion

The major results obtained in the present work have been summarized in Table V. The various combinations of ligands are arranged as a function of the corresponding extents of coupling to methioninol or methionine binding. The extents of coupling are also expressed as free energies of coupling. The table attempts to correlate the data with the charge distribution which is introduced by ligands into the active site. Tentative enumeration and localization of the phosphate charges are based on several assumptions which are indicated in the legend to the table and which will be discussed below.

It turns out from this and previous reports (Blanquet et al., 1975a,b) that synergistic coupling between ligands within the catalytic site of methionyl-tRNA synthetase requires the presence of the side chain of the specific amino acid, whereas the adenosine moiety, the amino acid carboxylate, or the phosphate groups are not requisites. This is particularly illustrated by the behavior of glycine which does not promote any of the synergistic phenomena. Indeed, glycine does not appear to bind the enzyme. Similar conclusions have been reached in the case of several other synthetases (Holler et al., 1975). Hence, binding to the synthetases of their specific amino acid would be the prerequisite of specificity which controls the activity of the enzyme site for further coupling (and catalysis, as we will discuss).

Once bound to the enzyme, the amino acid side chain (i.e., methioninol for instance) can couple as well with the binding of adenosine as with the binding of pyrophosphate. Among these couplings, the primary coupling involving the subsites for adenosine and methioninol (or analogues of methionine lacking the carboxylate group) corresponds to a free energy of interaction, $\Delta G = 0.8$ kcal, which is enhanced by the carboxyl function of the amino acid or the α-phosphoryl group of 5′-adenosine nucleotides. Upon introduction of the carboxylate, the gain in Gibbs free energy of interaction for adenosine binding is 0.3 kcal. Reciprocally the association of a 5′-adenosine nucleotide (ATP, ADP, or AMP) to the enzyme-bound methioninol (which lacks the reacting carboxylate) is clearly more strongly coupled than that of adenosine. In these cases, the gain in Gibbs free energy of interaction with respect to adenosine ranges from 1 to 2.2 kcal depending on the nucleotide considered and the presence of magnesium. These observations indicate that manifestation of major synergistic coupling requires the presence of one of the negatively charged groups which are involved in adenylate synthesis. This group may be either the carboxylate of methionine or the α-phosphate of the 5′-adenosine nucleotides. A working hypothesis for this behavior has been suggested previously on the basis of binding experiments with isoleucyl-tRNA synthetase (Holler et al., 1973). Amino groups of amino acid or amino alcohol are assumed to interact with and to open an ion pair, the positively charged component of which would further interact with the carboxylate or α-phosphate of amino acid or nucleotide. This hypothesis emphasizes the importance of the amino group of amino acids in their binding to specific synthetases (for a review see Kisselev and Favorova, 1974).

Direct evidence that the carboxylate and α-phosphate groups are directed toward a common enzymic locus is provided by the antagonistic couplings exerted on methionine binding either by ADP or AMP. The basis for this effect may be given in terms of electrostatic repulsion within the site between carboxylate and α-phosphate or in terms of competition (or overlap) of both groups for a common locus. The free energy ensuing from coupling between methionine and the adenosine part of these nucleotides is then not enough in order to overcome the electrostatic or geometric destabilization of the bound ligands. In contrast ATP-Mg^{2+}, which has an intact β-γ moiety, binds the enzyme-methionine complex (prior to the adenylate reaction) with no apparent coupling (Hyafil et al., 1976). This behavior introduces the coupling exerted by the pyrophosphate-magnesium moiety of the ATP molecule. Evidence for such a coupling is provided by the prominent effect of PP-Mg^{2+} on the stability of the enzyme/methionine/adenosine complex. Upon introduction of PP-Mg^{2+}, the free-energy gain on methionine binding (in the presence of adenosine) is equal to 3.4 kcal. Occupation of their sites by these ligands simulates an enzyme/methionine/ATP-Mg^{2+} complex lacking the reacting α-phosphate group. In this context, the synergistic and antagonistic couplings with methionine which follow, respectively, from the adenosine plus pyrophosphate-magnesium moieties and from the α-phosphate

TABLE V: Correlation of the Extents of Coupling with the Distribution of the Negative Charges Introduced by Ligands within the Active Site of Trypsin-Modified Methionyl-tRNA Synthase.[a]

Combination of ligands	Negative charges introduced into the: Amino acid site R–COO^-	ATP site: Ado–O–P(α)–O–P(β)–O–P(γ)–O^-	C	ΔG (kcal/mol)
		α / β / γ		
AAol + ADP		$1e^-$ $2e^-$	150	3
AAol + AMP + PP–Mg^{2+}		$2e^-$ $1e^-$ $1e^-$	78	2.6
AAol + ATP–Mg^{2+}		$1e^-$ $1e^-$	62	2.5
AAol + ADP–Mg^{2+}		$1e^-$ $1e^-$	26	1.95
AAol + Ado + PP		$1.5e^-$ $1.5e^-$	25.4	1.94
AAol + ATP		$1e^-$ $1e^-$ $2e^-$	18.5	1.75
AAol + AMP		$2e^-$	19.5	1.78
AAol + Ado + PP–Mg^{2+}		$(1e^-)$ $1e^-$	18	1.73
AAol + Ado			3.8	0.79
AAol + AMP + PP		$2e^-$ $1.5e^-$ $1.5e^-$	~1	~0
AA + Ado + PP–Mg^{2+}	$1e^-$	$1e^-$ $1e^-$	300	4.5
AA + Ado	$1e^-$		6	1.1
AA + ATP–Mg^{2+}	$1e^-$	$1e^-$ $1e^-$	~1	~0
AA + AMP	$1e^-$	$2e^-$	<1	<0
AA + ADP	$1e^-$	$1e^-$ $2e^-$	<1	<0

[a] The couplings, C, are respectively defined in the cases of methioninol and methionine by K_{AAol}/K_{AAol}^{X} and K_{AA}/K_{AA}^{X}, where X, the upper index of the equilibrium constants, indicates the combination of ligands which saturate the enzyme prior to its titration by AAol or AA. The corresponding Gibbs free energies of interaction are calculated as $\Delta G = +RT \log C$ at 25 °C. Enumeration and localization of the negative charges introduced into the active site by the ligands are based on the following premises. (1) The charges associated to phosphate groups are expressed as electrons and calculated according to their pK in solution. Upon binding by the magnesium ion, the negative charges of phosphate groups are neutralized. According to Cohn and Hughes (1962), we assume that, in solution, ATP and ADP coordinate the magnesium ion respectively via the β and γ and via the α and β phosphoryl groups. In the absence of relevant studies, we assume that the divalent ion binds 5′-AMP in solution via chelation of the α-phosphate group. We have verified by pK measurements that binding of magnesium to the phosphate groups of 5′-AMP, ADP, and ATP does not modify the extent of ionization of the remaining acidic groups (phosphate groups and N-1 position). Therefore, these groups are fully ionized in our buffer conditions. In the case of pyrophosphate, we have determined the pK values of the acidic groups in water, with or without magnesium. In the absence of magnesium, three acidic groups of the pyrophosphate molecule are fully ionized in our buffer conditions (pKs are <2, <2, and 6.1) while the fourth hydroxyl group (pK = 8.75) is not significantly ionized. In the presence of magnesium, all of the acidic groups are now ionized (both pKs 6.1 and 8.75 become equal to 6.0). In this case magnesium is assumed to bind both phosphate groups of pyrophosphate. (2) It is assumed that the action of magnesium within the enzyme site is restricted to the β–γ subsites of the ATP site. (3) Pyrophosphate may bind the α–β subsite of the ATP site provided that the carboxylate group of methionine and/or the α phosphate of 5′-AMP are absent; otherwise pyrophosphate binding is directed toward the β–γ subsite. (4) In the table no attention is given to the problem of steric overlaps between oxygen atoms within the site.

group of ATP–Mg^{2+} would compensate each other, thus leading to the apparent absence of coupling. In accordance with this hypothesis, the methioninol plus ATP–Mg^{2+} and the methioninol plus AMP and PP–Mg^{2+} combinations which simulate an enzyme/methionine/ATP–Mg^{2+} complex, this time lacking the reacting carboxylate group, belong to the class of the highest couplings in Table V. At this stage, it may be noted that each of the latter combinations develops almost identical couplings on methioninol binding to trypsin-modified methionyl-tRNA synthetase (ΔG = 2.6–2.5 kcal) in spite of the split nature of the AMP + PP–Mg^{2+} combination. Indeed, the split introduces one more oxygen and two more negative charges within the site as compared to intact ATP–Mg^{2+}. In the absence of magnesium, such electrostatic and/or steric hindrances definitely play in favor of ATP during its coupling process with methioninol binding (see Table V). Therefore we must admit that within the site, magnesium may rearrange or adjust the charge and geometry of AMP plus PP–Mg^{2+}. In this context the dead-end enzyme/methioninol/AMP/PP–Mg^{2+} complex would simulate better, in terms of charge and geometry, the reacting enzyme/methionyl~AMP/PP–Mg^{2+} complex which we know from stopped-flow studies resembles closely the enzyme/methionine/ATP–Mg^{2+} complex with respect to free energy (Hyafil et al., 1976).

Magnesium plays an important role in developing the couplings of the pyrophosphate–magnesium moiety. With the methionine/adenosine/pyrophosphate, methionine/pyrophosphate, and methioninol/AMP/pyrophosphate combinations magnesium is required to promote the coupling for which pyrophosphate is responsible. Also, magnesium increases markedly the coupling in the case of the methioninol/ATP combination. It must be noted that in these couplings either the carboxylate or the α-phosphate is present. The only instances in which PP may act synergistically in the absence of magnesium is when both the carboxylate and α-phosphate groups are deleted. This is the case of the methioninol/pyrophosphate and methioninol/adenosine/pyrophosphate combinations. In these combinations, the question remains open if pyrophosphate binds the β–γ or the α–β subsites of the ATP site on the enzyme. The latter case would resemble a situation where the α-phosphate charge is present within the site, thus triggering major coupling on methioninol binding. For example, the methioninol/adenosine/pyrophosphate combination would simulate the methioninol/ADP combination. It is striking to note that in both these cases magnesium reduces the extent of synergistic coupling. This could be achieved by chelating the crucial charge which we assume is directed toward the catalytic locus and/or by directing the pyrophosphate

molecule binding to the β-γ subsite with concomitant loss of the charge at the α position. The latter alternative supports the idea that binding of magnesium within the catalytic locus is favored at the β-γ subsite of the ATP site.

To conclude with the role of magnesium, we have to inspect the effect of the ion on the binding parameters of 5′-nucleotides and pyrophosphate to the free enzyme (enzyme/adenosine complex in the case of pyrophosphate). In all cases, the metal decreases the affinity of the enzyme for the ligand under study (Tables II and III). In other words, this reflects a better affinity of the enzyme for the ligand noncomplexed to magnesium. In contrast, magnesium improves the binding of the same ligands if they are involved in a coupling process. At this time, this reinforcing effect of metal ion, which implies binding to the catalytic site, requires that polyphosphoryl groups are engaged within the catalytic center of methionyl-tRNA synthetase. The ion does not interfere with the couplings involving methionine, methioninol, or 5′-AMP which do not satisfy the polyphosphoryl requirement (i.e., involvement of at least the β subsite of the ATP site).

Favorable binding of magnesium to the enzyme complexes via the β-γ subsites of the ATP site would be therefore triggered by the following events: enzymic rearrangement upon binding of the amino acid side chain, opening of an ion pair by the ammonium group of the amino acid and by one of the carboxylate or α-phosphate reacting groups, improved binding of the adenosine moiety, and occupation of the β-γ subsites of the ATP site. Then the metal ion is attracted within the site and might lock the dead-end synergistic enzyme complexes which we have displayed. It is reasonable to assume that the divalent ion may also lock the transient enzyme/methionine/ATP-Mg^{2+} and enzyme/methionyl adenylate/PP-Mg^{2+} complexes which we have shown to occur during the course of methionyl adenylate synthesis (Hyafil et al., 1976). Pyrophosphate-Mg^{2+} binding is indeed coupled to the enzyme-bound methionyl adenylate. Thus, arising first of all from the specific binding of the amino acid are a series of self-amplifying coupled binding processes which take advantage of magnesium, the cofactor of catalysis. The resulting free energy which is not observed at the level of the methionine/ATP-Mg^{2+} coupling may be utilized directly to pay for the destabilization of bound substrates relative to the transition state (Jencks, 1975). Such destabilization is assumed to increase the reaction rate if it is relieved upon formation of the transition state.

Acknowledgments

This work was carried out in the laboratory of Dr. J. P. Waller, whose continuous support and stimulating criticism are gratefully acknowledged. The authors are also indebted to Dr. M. Gueron for fruitful discussions and critical reading of the manuscript.

References

Baldwin, A. N., and Berg, P. (1966), *J. Biol. Chem. 241,* 839-895.

Belaich, J. P., and Sari, J. C. (1969), *Proc. Natl. Acad. Sci. U.S.A. 64,* 763-770.

Blanquet, S., Fayat, G., Poiret, M., and Waller, J. P. (1975a), *Eur. J. Biochem. 51,* 567-571.

Blanquet, S., Fayat, G., and Waller, J. P. (1974), *Eur. J. Biochem. 44,* 343-351.

Blanquet, S., Fayat, G., and Waller, J. P. (1975b), *J. Mol. Biol. 94,* 1-15.

Blanquet, S., Fayat, G., Waller, J. P., and Iwatsubo, M. (1972), *Eur. J. Biochem. 24,* 461-469.

Blanquet, S., Iwatsubo, M., and Waller, J. P. (1973), *Eur. J. Biochem. 36,* 213-226.

Cassio, D., Lemoine, F., Waller, J. P., Sandrin, E., and Boissonnas, R. A. (1967), *Biochemistry 6,* 827-835.

Cassio, D., and Waller, J. P. (1971), *Eur. J. Biochem. 20,* 283-300.

Cohn, M., and Hughes, T. R. (1962), *J. Biol. Chem. 237,* 176-181.

Fayat, G., and Waller, J. P. (1974), *Eur. J. Biochem. 44,* 335-342.

Fersht, A. R., and Kaethner, M. M. (1976), *Biochemistry 15,* 3342-3346.

Holler, E., Hammer-Raber, B., Hanke T., and Bartmann, P. (1975), *Biochemistry 14,* 2496-2503.

Holler, E., Rainey, P., Orme, A., Bennett, E. L., and Calvin, M. (1973), *Biochemistry 12,* 1150-1159.

Hopfield, J. J. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4135-4139.

Hopfield, J. J., Yamane, T., Yue, V., and Coutts, S. M. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 1164-1168.

Hyafil, F., and Blanquet, S. (1977), *Eur. J. Biochem.* (in press).

Hyafil, F., Jacques, Y., Fayat, G., Fromant, M., Dessen, P., and Blanquet, S. (1976), *Biochemistry 15,* 3678-3685.

Jencks, W. P. (1975), *Adv. Enzymol. 43,* 219.

Kisselev, L. L., and Favorova, O. O. (1974), *Adv. Enzymol. 40,* 141-238.

Kosakowski, H. M., and Holler, E. (1973), *Eur. J. Biochem. 38,* 274-282.

Lawrence, F., Shire, D., and Waller, J. P. (1974), *Eur. J. Biochem. 41,* 73-81.

Loftfield, R. B., and Vanderjagt, D. (1972), *Biochem. J. 128,* 1353-1356.

Ninio, J. (1975), *Biochimie 57,* 587-595.

von der Haar, F., and Cramer, F. (1976), *Biochemistry 15,* 4131-4138.