The Catalytic Mechanism of Amino Acid:tRNA Ligases. Synergism and Formation of the Ternary Enzyme–Amino Acid–ATP Complex†

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ABSTRACT: Formation of binary and ternary enzyme–ligand complexes was investigated for amino acid:tRNA ligases specific for L-isoleucine, L-leucine, and L-phenylalanine. Each of the enzymes exhibited synergistic binding when a substrate was substituted by a structurally related compound. The strength of coupling between the sites binding the amino acid and ATP was strongly dependent on the structure of ligands. The phenomenon was observed with the L-leucine- and L-phenylalanine-specific enzymes only in the presence of magnesium. Spermine was inhibitory for L-phenylalanine:tRNA ligase. From the variation with structure of the strength of the observed synergism a correlation scheme was derived considering the ammonium group, the carboxylate group and the side chain of the amino acid, and the adenosine and triphosphate moieties of ATP. The strength of coupling between the subsites binding various combinations of these moieties was evaluated. We found that binding of the subgroups of the amino acid exerts an intramolecular synergism. The strength of this intramolecular synergism was similar to the strength of the intermolecular synergism observed for the simultaneous binding of an amino alcohol and ATP (or $MgATP^{2-}$). We have derived a molecular mechanism for the formation of the ternary enzyme–amino acid–ATP (or $MgATP^{2-}$) complex taking into account the synergistic phenomena. The complex is considered to involve electrostatic repulsion between the amino acid carboxylate and the ATP triphosphate moieties. When one of the negatively charged groups has been eliminated, the enzymic rearrangement which facilitates the formation of this complex may be seen as a synergistic coupling.

The primary reaction during catalysis of tRNA aminoacylation in the presence of amino acid:tRNA ligases probably involves the synthesis of a reversibly enzyme-bound aminoacyl adenylate from the substrates amino acid and ATP (review by Kisselev, 1974, and references therein). The dynamics of the association between substrates and enzyme prior to the formation of adenylate have been investigated for L-isoleucine:tRNA ligase (Holler and Calvin, 1972). Binding of L-isoleucine was found to follow a two-step kinetic process probably involving the rearrangement of a primary enzyme–amino acid complex. Further investigations with L-isoleucine related compounds revealed that enzyme binding of the amino acid and ATP may not necessarily proceed independently (Holler et al., 1973). We have recently reported a similar observation for L-phenylalanine:tRNA ligase (Kosakowski and Holler, 1973). Ligands which were structurally related to amino acid and ATP were shown to bind via a random mechanism to the enzymes, exhibiting cooperative formation of the ternary complexes. In the present publication we shall analyze the contributions of the structural components of the amino acid and of ATP to the enhancement of binding affinity of either ligands during their simultaneous association with the amino acid:tRNA ligase.

Materials and Methods

Preparation of Enzyme. L-Phenylalanine:tRNA ligase (specific activity 53,600 nmol mg^{-1} hr^{-1}) and L-isoleucine:tRNA ligase (specific activity 16,500 nmol mg^{-1} hr^{-1}) were prepared from *Escherichia coli* KIO in the presence of phenylmethanesulfonyl fluoride as described by Hanke et al. (1974) and by Baldwin and Berg (1966), respectively, with slight modifications. L-Leucine:tRNA ligase had been enriched from *E. coli* B (specific activity 3000 nmol mg^{-1} hr^{-1}) and was a gift from Drs. A. Mehler and F. Chapeville. tRNA used in aminoacylation reactions was unfractionated and had been prepared according to Zubay (1962).

Chemicals. Radioactive amino acids (specific radioactivities ranging from 340 to 450 mCi/mol) were purchased from Radiochemical Centre (Amersham). 6-*p*-Toluidinylnaphthalene-2-sulfonate, potassium salt (TNS),[1] was obtained from Serva (Heidelberg), L-phenylalaninol from Fluka (Buchs), L-isoleucinol from Cyclo Chemical (Los Angeles), 3-methyl-1-pentanol from Aldrich (Milwaukee), and ATP from Boehringer (Mannheim). All other chemicals were analytical grade and purchased from Merck (Darmstadt).

L-Leucinol hydrochloride (melting point 150°) was synthesized according to Vogl and Pöhm (1952). All amino acids and amino alcohols were homogeneous by thin-layer chromatography on silica gel (Merck) using 1-butanol satured with 20% acetic acid and benzene–pyridine–acetic acid (80:20:5, v/v) as solvents and ninhydrin (spray, Merck) as detecting reagent. ATP was homogeneous on polyethylenimine thin-layer chromatography (Polygram CEL 300 PEI from Machery-Nagel, Düren) using 0.75 *M* potassium phosphate (pH 3.5) (adjusted with HCl) as solvent.

Aminoacylation of tRNA. Enzyme activity was determined by the tRNA-aminoacylation assay at pH 7.5 and 28° as described previously for L-phenylalanine:tRNA ligase (Kosakowski and Böck, 1970) with the exception that

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[1] Abbreviation used is: TNS, 6-*p*-toluidinylnaphthalene-2-sulfonate, potassium salt.

Table I: Formation of Ternary Amino Acid:tRNA Ligase Complexes with ATP and Amino Acid or Amino Alcohol.[a]

Amino Acid:tRNA Ligase	Ligand 1	Ligand 2	K_2 (M)	K_{12} (M)	K_2/K_{12}
L-Leucine[b]	$MgATP^{2-}$	L-Leucinol	4.0 (0.50) × 10^{-3}	6.1 (54) 10^{-5}	65 (1.0)
	$MgATP^{2-}$	L-Leucine	1.5 (3.6) × 10^{-4}	1.2[e] (3.5) × 10^{-4}	1.2 (1.0)
L-Isoleucine[c]	$MgATP^{2-}$	L-Isoleucinol	6.5 (5.5) × 10^{-3}	3.3 (4.8) × 10^{-6}	2000 (1100)
	$MgATP^{2-}$	L-Isoleucine	5.8[j] (5.8[i]) × 10^{-6}	4.0[f] (2.0[j]) × 10^{-6}	1.4 (2.9)
L-Phenylalanine[d]	$MgATP^{2-}$	L-Phenylalaninol	1.2[g] (0.23) × 10^{-3}	4.0[g] (200) × 10^{-6}	300 (1.1)
	$MgATP^{2-}$	L-Phenylalaninol	1.87 × 10^{-3}[k]	1.3 × 10^{-5}[k]	144
	$MgATP^{2-}$	L-Phenylalanine	3.0[g] (5.0) × 10^{-5}	5[h] (5.0) × 10^{-5}	0.6 (1.0)

[a] Titration experiments at 25°, 0.05 M Tris-HCl (pH 7.5), 0.1 mM EDTA, and 0.2 mM dithioerythrol in the presence of TNS as a reporter group. Magnesium was present as indicated separately. Values in parentheses refer to the absence of magnesium. Enzyme concentration varied between 0.1 and 0.8 μM. Fluorescence was measured and evaluated as described previously (Holler et al., 1971; Kosakowski and Holler, 1973). Excitation wavelengths were either 300 nm (mainly Trp absorbance) or 366 nm (TNS). Emission was recorded at 445 nm. At conditions where excitation was set to 300 nm, energy transfer from intrinsic protein–Trp to TNS was involved (Holler et al., 1971). [b] 10 mM $MgCl_2$–10 μM TNS. [c] 10 mM free $MgCl_2$–20 μM TNS. [d] 5 mM free $MgCl_2$–4 μM TNS. [e] ATP-PP_i exchange, Rouget and Chapeville (1970). [f] Cole and Schimmel (1970). [g] Kosakowski and Holler (1973). [h] Santi et al. (1971). [i] Calvin and Holler (1971). [j] Calvin and Holler (1972). [k] Conditions as under *a* and *d* except that KCl at a concentration of 0.5 M had been added.

the concentration of tRNA was doubled when the formation of L-isoleucyl-tRNA was measured.

Fluorescence Titration. Fluorescence titration experiments were performed using 6-*p*-toluidinylnaphthalene-2-sulfonate as a reporter group following the procedures outlined previously for L-isoleucine:tRNA ligase and L-phenylalanine:tRNA ligase (Holler et al. 1971; Kosakowski and Holler, 1973). The same procedure could be applied to L-leucine:tRNA ligase even though the enzyme was not a homogeneous preparation. From the independence of the dissociation constants for L-leucine and ATP against varied concentration of the dye (2–40 μM) it may be concluded that the fluorescent reporter group binds to the enzyme in a noncompetitive mode. It should be mentioned that, when using this titration procedure, we could not detect the tight binding of ATP reported by Rouget and Chapeville (1971). Possibly, the site of tight binding is not at the active site.

Evaluation of the titration experiments was based on the formalism presented in a previous paper (Kosakowski and Holler, 1973). We have denoted the dissociation constant of a ligand from a binary complex as K_2 and from a ternary complex as K_{12}. In general, this dissociation constant can be obtained by titration of a mixture containing enzyme and saturating concentrations of the co-ligand (denoted as first ligand) with the ligand under consideration (denoted as second ligand). In cases where saturating concentrations of the first ligand could not be prepared the order of ligand addition was reversed: enzyme was preincubated with the second ligand at a nonsaturating concentration, and the mixture then titrated with the first ligand. The apparent dissociation constant $K_{(L)}$ evaluated from the titration curve was used to calculate the dissociation constant K_{12} on the basis of the following equation (Kosakowski and Holler, 1973):

$$K_{12} = \frac{[\text{ligand}_2]_0 K_{(L)}}{K_1 \frac{K_2 + [\text{ligand}_2]_0}{K_2} - K_{(L)}} \quad (1)$$

The symbol K_1 refers to the dissociation constant of the binary complex between enzyme and the first ligand, subscript zero indicates total concentration.

The values given for dissociation constants represent averages from two to four measurements. Standard experimental deviations are in the order of 10–20%, those calculated for K_{12} sometimes in the order of 50%.

Results

Synergistic Coupling for Amino Acid:tRNA Ligases. The stability of an amino acid:tRNA ligase complex with an amino alcohol may be strongly enhanced in the presence of ATP (or $MgATP^{2-}$) as a second ligand binding to the enzyme (Kosakowski and Holler, 1973). An enhancement is seen in Table I as a 65- to 2000-fold decrease in the value of the dissociation constant for the amino alcohol. The ratio K_2/K_{12} (the value of the dissociation constant in the absence of a second ligand over the dissociation constant in the presence of saturating concentration of a second ligand) is taken as a measure of the interrelation between the binding of the second ligand and the binding of the first ligand. The free energy of the interrelation (from here on called briefly "coupling") is related to the ratio of dissociation constants by $\Delta F_{\text{coupl.}} = -RT \ln (K_2/K_{12})$. In the present paper we shall refer to the value of this ratio as the strength of coupling.

From Table I the following observations emerge. (1) Synergistic coupling was obtained with each enzyme, the strength of coupling being smallest for the L-leucine-specific enzyme and largest for the L-isoleucine-specific enzyme. (2) Magnesium was required for the coupling to occur with the L-leucine- and L-phenylalanine-specific enzymes but not with the L-isoleucine-specific enzyme. (3) Synergistic coupling was abolished when the amino acid was used instead of the amino alcohol as the other ligand. This observation suggests that coupling must depend on the structure of the ligands. (4) For all the enzymes, the amino acid was bound considerably more strongly than was the amino alcohol. It will be of interest to see whether this increase of complex stability can be ascribed to a synergistic coupling accompanying the binding of the carboxylate and the remainder of the amino acid to particular subsites of the enzymes (intramolecular synergism) thereby replacing the coupling observed between the amino alcohol and ATP (or $MgATP^{2-}$) (intermolecular coupling).

Magnesium Requirement. The magnesium ion requirement which was observed for synergistic coupling was paralleled by effects of the metal ion on the formation of binary enzyme–ligand complexes with the amino alcohols (Table I) and with ATP (Table II). While in the presence of magnesium the dissociation constants (K_2) for the amino alcohols were shifted to appreciably higher values, those for the binding of ATP were shifted to either higher or lower values depending on the enzyme.

Table II: Amino Acid:tRNA Ligase Complexes with ATP in the Presence of Magnesium[a]

Enzyme Specificity	Dissociation Constant	
	ATP[f] (M)	$MgATP^{2-}$ (M)
L-Phenylalanine	6×10^{-3}[b]	6×10^{-4}[c]
L-Leucine	2.9×10^{-4}	1.3×10^{-3}[c]
L-Isoleucine	1.75×10^{-4}[d]	2.5×10^{-4}[e]

[a] Unless otherwise mentioned, conditions are as under Table I. [b] Determined from competition experiments in the presence of adenosine and L-phenylalanine, as described (Kosakowski and Holler, 1973). [c] 10 mM free $MgCl_2$. [d] Holler et al. (1971). [e] 5 mM free $MgCl_2$. [f] No magnesium.

When magnesium was substituted by spermine, complete cancellation of the synergism was observed for the magnesium-dependent L-phenylalanine:tRNA ligase but not for the magnesium-independent L-isoleucine:tRNA ligase (Table III). In contrast, the substitution had little effect when the coupling for the binding of adenosine and L-phenylalaninol was measured.

The effect of altering the ionic strength was minor, as may be seen from Table I (footnotes g and k).

Structural Requirement. Our previous experience with the coupling which accompanies ligand binding to amino acid:tRNA ligases (Holler et al., 1973; Kosakowski and Holler, 1973) suggested that we investigated the involvement of electrostatically charged and neutral moieties of the amino acid and ATP. Thus the amino acid was divided into the specific side chain, the ammonium group, and the carboxylic group. ATP was divided into adenosine and the triphosphate moiety.

The goal of the investigation was now to determine the strength of the coupling between the binding reactions of the various combinations of the ligand subgroups to (hypothetical) enzymic subsites. The various combinations are pictured in Figure 1. Structural components, which can be connected by a solid line between any two of them, can be members of a combination. Thus, a combination may exist of up to four components. To include our findings that the binding of an amino acid and ATP did not exhibit synergism, we have chosen a tetrahedral bipyramid in order to exclude combinations containing the carboxylate and triphosphate groups simultaneously.

An evaluation of the strength of coupling pertaining to the various combinations was attempted as follows from the values of the dissociation constants listed in Tables I, IV, and V. Benzyl alcohol and 3-methyl-1-pentanol were chosen to represent the side chain of L-phenylalanine and L-isoleucine, respectively. Dihydrocinnamic acid resembled the L-phenylalanine side chain together with the carboxylate group, L-phenylalaninol the side chain together with the ammonium group, glycine the ammonium group together with the carboxylate group, etc. In some cases the triphosphate moiety was tentatively replaced by pyrophosphate.

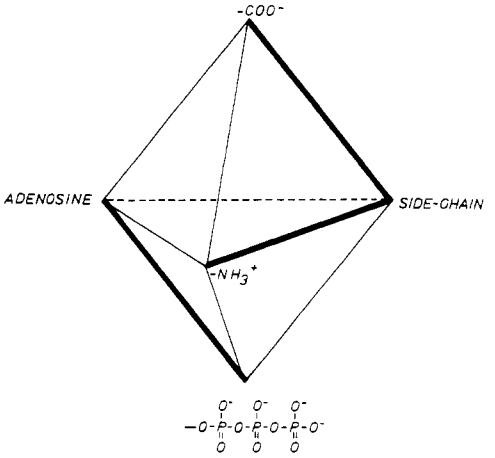


FIGURE 1: A scheme indicating the various combinations of the structural components of an amino acid and ATP in order to exhibit synergism during binding to an amino acid:tRNA ligase. A combination is possible with components where two of them are connected by a solid line. For more details see text.

The smallest possible combinations consist of two structural components like the combinations side chain/adenosine or side chain/ammonium group. According to our results, binding of benzyl alcohol or 3-methyl-1-pentanol was unaffected by the presence of adenosine (Tables IV and V), indicating that coupling does not exist between binding of the side chain and adenosine. The values of the dissociation constants (K_2) for benzyl alcohol, dihydrocinnamic acid, phenylethylamine, and L-phenylalaninol are very similar (Table IV), indicating that the ammonium group and the carboxylate group were without effects on the binding of the side chain. Thus, there appears to be no coupling for the combinations side chain/ammonium group and side chain/carboxylic group. The same conclusion may be drawn for the L-isoleucine system (Table V). The lack of binding of the ammonium ion, of the carbonate ion, and of glycine at concentrations as high as 0.1 M (as judged by the fluorescence technique) indicated that the ammonium group, the carboxylic group, and the combination of both bound considerably weaker than the amino acid specific side chain. The question whether simultaneous binding of the ammonium group and the carboxylic group exerts synergism cannot be answered at present.

In some measurements we have used pyrophosphate in

Table III: The Effect of Spermine on Synergistic Coupling.[a]

Enzyme	Cation	Ligand 1	Ligand 2	K_2 (M)	K_{12} (M)	K_2/K_{12}
L-Phenylalanine:tRNA ligase	Magnesium[b]	Adenosine	L-Phenylalanine	3×10^{-5}	0.16×10^{-5}	19
	Spermine[c]	Adenosine	L-Phenylalanine	2.4×10^{-5}	0.38×10^{-5}	6.3
	Spermine[c]	Spermine–ATP[d]	L-Phenylalanine	2.4×10^{-5}	2.1×10^{-5}	1
	Magnesium[b]	$MgATP^{2-}$	Phenylethylamine	3.4×10^{-3}	0.07×10^{-3}	50
	Spermine[c]	Spermine–ATP[d]	Phenylethylamine	2.3×10^{-3}	1.2×10^{-3}	2
L-Isoleucine:tRNA ligase	Magnesium[b]	$MgATP^{2-}$	L-Isoleucinol	6.5×10^{-3}	3.3×10^{-6}	2000
	Spermine[c]	Spermine–ATP[e]	L-Isoleucinol	$\geq 5.5 \times 10^{-3}$	0.03×10^{-3}	≥ 100

[a] Fluorescence titration at 25°, 0.05 M Tris-HCl (pH 7.5), 0.1 mM EDTA, and 0.1 mM dithioerythrol in the presence of TNS as indicated under Table I. [b] 10 mM concentration of magnesium. [c] In the presence of 2.0 mM spermine–ATP and 0.5 mM free spermine. [d] Equimolar amounts of ATP and spermine. [e] Holler (1973).

Table IV: Structural Requirements for Coupling of Ligand Binding with L-Phenylalanine:tRNA Ligase.[a]

Ligand 1	Ligand 2	K_2 (*M*)	K_{12} (*M*)	K_2/K_{12}
MgATP	Benzyl alcohol	4.4×10^{-3}	0.34×10^{-3}	13
Adenosine	Benzyl alcohol		4.0×10^{-3}	1.1
MgATP	Dihydrocinnamic acid	3.9×10^{-3}	7.8×10^{-3}	0.5
Adenosine			3.2×10^{-3}	1.2
MgATP	Phenylpropiolic acid	1.7×10^{-3}	2.5×10^{-3}	0.7
MgATP	Benzylamine	1.1×10^{-3}	0.7×10^{-3}	1.6
MgATP	Phenylethylamine	3.4×10^{-3}	0.1×10^{-3}	34
Adenosine	Phenylethylamine		0.5×10^{-3}	7
PP_i	Phenylethylamine		6.8×10^{-4}	5
MgATP	L-Phenylalaninol	1.2×10^{-3}	4×10^{-6}	300
Adenosine	L-Phenylalaninol		0.12×10^{-3}	10
Adenosine	L-Phenylalanine	3.0×10^{-5}	2.9×10^{-6}	10
PP_i			4.0×10^{-5}	0.8

[a] Conditions were as under Table I, footnote *d*.

Table V: Structural Requirements for Coupling of Ligand Binding with L-Isoleucine:tRNA Ligase.[a]

Ligand 1	Ligand 2	K_2[b] (*M*)	K_{12}	K_2/K_{12}
Adenosine	L-Isoleucine	5.8×10^{-6}	6.0×10^{-6}	1
Adenosine	L-Isoleucinol	5.5×10^{-3}	5.0×10^{-3}	1.1
PP_i	L-Isoleucinol	5.5×10^{-3}	3.1×10^{-5}	180
Adenosine *plus* PP_i	L-Isoleucinol	5.5×10^{-3}	2.4×10^{-5}	230
Adenosine	3-Methyl-1-pentanol	2.5×10^{-3}	2.0×10^{-3}	1.3
PP_i	3-Methyl-1-pentanol	2.5×10^{-3}	2.5×10^{-3}	1
Adenosine *plus* PP_i	3-Methyl-1-pentanol	2.5×10^{-3}	2.0×10^{-3}	1.3
ATP	3-Methyl-1-pentanol	2.5×10^{-3}	2.2×10^{-4}	11.5

[a] Titration experiments were performed in the absence of magnesium. Conditions (in the presence of 20 μM TNS) were those for (*a*) under Table I. [b] Taken from Holler et al. (1973).

order to mimic the triphosphate moiety. This had the advantage that binding of pyrophosphate could be measured separately. We are aware, of course, that the effects measured in the presence of this anion could only be an approximation of those to be observed in the presence of the triphosphate moiety. With pyrophosphate, coupling was not observed in the presence of 3-methyl-1-pentanol (0.02 *M*), ammonium chloride (0.1 *M*), or adenosine (Kosakowski and Holler, 1973). Being aware of the incompleteness of our experimental information, we tentatively conclude that most or all of the binary combinations do not exhibit synergism.

In a following step we have evaluated the strength of coupling which accompanied the binding of three structural components. In terms of the tetrahedral bipyramid (Figure 1), three components determine a particular triangle. Each is characterized by a unique strength of coupling. The various combinations are summarized for the L-phenylalanine- and L-isoleucine-specific enzymes in Tables VI and VII. Computation was based on the previous tentative conclusion that binary combinations did not exhibit synergism. Thus, the strength of coupling occurring upon simultaneous binding of the components adenosine/ammonium group/side chain was assumed to be identical with the strength of coupling measured for the binding of an amino alcohol together with adenosine (Tables IV and V). In the case of the combination ammonium group/side chain/carboxylic group resembling L-phenylalanine, several ways of evaluation were explored (Table V). Within a factor of 3 the same results were obtained supporting the validity of our assumptions. We have also evaluated the strength of coupling for combinations which contained pyrophosphate instead of the triphosphate moiety (Tables V and VI).

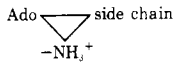
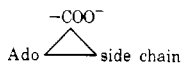
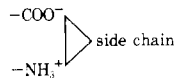
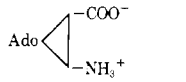
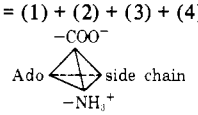
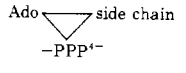
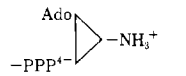
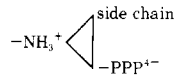
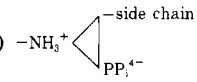
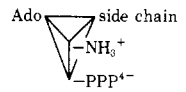
As is seen from Tables V and VI, the strength of coupling could not be measured for all of the triangular combinations, however, it may be computed under certain assumptions to be considered later.

The strength of the synergism to be obtained for simultaneous binding of the tetrahedral combinations adenosine/ammonium group/side chain/carboxylic group and adenosine/ammonium group/side chain/triphosphate moiety was determined by comparing the values of the dissociation constants pertaining to binding of the amino alcohols with the values for binding of the amino acids in the presence of adenosine, and by comparing the values of the dissociation constants in the absence and the presence of ATP (or $MgATP^{2-}$), respectively (Tables VI and VII). It was surprising to find the values for the two different tetrahedra to be of the same magnitude. This observation is valid for both enzymes. It is likely that coupling followed a similar molecular mechanism for both combinations.

The synergism for a particular tetrahedral combination is composed of the strength of coupling for the appropriate triangular combinations. Then, the free energy of coupling might be obtained as the summation of the free energies of coupling for the four triangular combinations. Because of the logarithmic relationship between binding constants and free energy of binding, the strength of the coupling for the tetrahedral set of components is then the product of the coupling pertaining to the triangular sets. We have applied this relation in order to calculate the strength of synergism where it could not be directly measured.

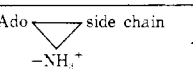
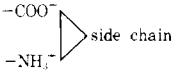
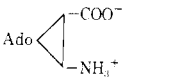
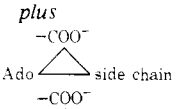
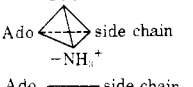
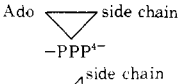
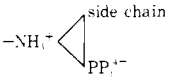
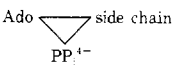
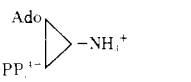
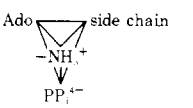
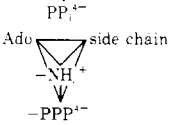
The following results were computed. (1) The combination adenosine/ammonium group/carboxylic group bound without synergism, calculated from the results for combinations 1, 2, 3, and 6, or via another route from the results for

Table VI: Strengths of Coupling for Triangular and Tetrahedral Combinations of Structural Components Interacting with L-Phenylalanine: tRNA Ligase.

Combination of Components	Method of Computation[a]	Strength of Coupling
(1) 	K(phenylalaninol)$_2$/K(Ado, phenylalaninol)$_{12}$	10
(2) 	K(dihydrocinnamate)$_2$/K(Ado, dihydrocinnamate)$_{12}$	1.2
(3) 	K(phenylalaninol)$_2$/K(Phe)$_2$ K(dihydrocinnamate)$_2$/K(Phe)$_{12}$ K(propiolic acid)$_2$/K(Phe)$_{12}$	40 130 57
(4) 	Coupling (6)/(coupling (1)·coupling (2)·coupling (3))	1
(5) = (1) + (2) + (4)	K(Phe)$_2$/K(Ado, Phe)$_{12}$	10
(6) = (1) + (2) + (3) + (4) 	K(phenylalaninol)$_2$/K(Ado, Phe)$_{12}$	415
(7) 	K(benzyl alcohol)$_2$/K(ATP, benzyl alcohol)$_{12}$	13
(8)  *plus*	Coupling (11)/(coupling (1)·coupling (7))	3
(9) 		
(10) 	K(phenylethylamine)$_2$/K(PP$_i$, phenylethylamine)$_{12}$	5
	K(phenylalaninol)$_2$/K(ATP, phenylalaninol)$_{12}$	300

[a] Values of dissociation constants K_2 and K_{12} are defined under Materials and Methods and were taken from Tables I and IV.

Table VII: Strengths of Coupling for Triangular and Tetrahedral Combinations of Structural Components Interacting with L-Isoleucine: tRNA Ligase.[a]

Combination of Components	Method of Computation[b]	Strength of Coupling
(1) 	K(isoleucinol)$_2$/K(Ado, isoleucinol)$_{12}$	1
(2) 	K(isoleucinol)$_2$/K(Ile)$_2$	950
(3) = (1) *plus*  *plus* 	K(Ile)$_2$/K(Ado, Ile)$_{12}$	1
(4) 	K(isoleucinol)$_2$/K(Ado, Ile)$_{12}$	920
(5) 	K(3-methyl-1-pentanol)$_2$/K(ATP, 3-methyl-1-pentanol)$_{12}$	12
(6) 	K(isoleucinol)$_2$/K(PP$_i$, isoleucinol)$_{12}$	180
(7) 	K(3-methyl-1-pentanol)$_2$/K(Ado + PP$_i$, 3-methyl-1-pentanol)$_{12}$	1.3
(8) 	Coupling (9)/(coupling (6)·coupling (7)·coupling (1))	1
(9) 	K(isoleucinol)$_2$/K(Ado + PP$_i$, isoleucinol)$_{12}$	230
(10) 	K(isoleucinol)$_2$/K(ATP, isoleucinol)$_{12}$	1100

[a] No magnesium present. [b] Values of the dissociation constants K_2 and K_{12} were taken from Tables I and V.

combinations 1, 2, and 5 in Table VI. This result for L-phenylalanine:tRNA ligase is probably also valid for L-isoleucine:tRNA ligase, because the combinations 3 in Table VII are already without synergism. (2) Again zero synergism was calculated for the combination adenosine/pyrophosphate/ammonium group (combination 8 of Table VII) with the L-isoleucine-specific enzyme. (3) Absence of synergism for the combination adenosine/triphosphate moiety/ammonium group with L-phenylalanine:tRNA ligase was concluded on the basis of the combinations 8, 9, and 10 in Table VI.

From the foregoing analysis the following conclusions were derived. (1) Coupling was observed with L-phenylalanine:tRNA ligase and L-isoleucine:tRNA ligase for the binding of the triangular combinations adenosine/triphosphate moiety/side chain, ammonium group/side chain/carboxylic group and ammonium group/pyrophosphate (and also probably the triphosphate moiety)/side chain. Synergism for adenosine/ammonium group/side chain was only seen for the L-phenylalanine-specific enzyme but not for the L-isoleucine-specific enzyme. Thus, coupling was only observed for triangular combinations which contain at least the side chain and one electrostatically charged group, in the case of L-isoleucine:tRNA ligase two electrostatically charged groups. (2) The coupling observed for the combination ammonium group/side-chain/carboxylic group occurs upon binding of the amino acid and may be called intramolecular synergism as opposed to the intermolecular synergism observed for the binding of an amino alcohol and ATP (or $MgATP^{2-}$). (3) Substitution of the triphosphate moiety by pyrophosphate leaves the synergistic reaction still functioning. A reduction of the strength of synergism was observed in the case of L-isoleucine:tRNA ligase.

We were also interested to see whether the synergistic coupling followed a characteristic enthalpy and entropy. Accordingly, the dissociation constants for L-phenylalaninol, L-phenylalanine, and L-phenylalaninol binding in the presence of $MgATP^{2-}$ have been measured as a function of the temperature (Figure 2). The dependence could be approximated by linear functions, and the enthalpies were determined from the slopes. The values are summarized in Table VIII. As a result, it was found that the coupling was

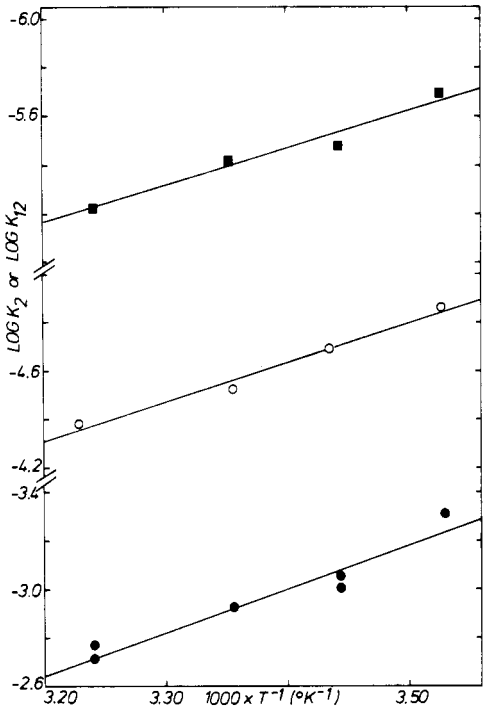


FIGURE 2: Temperature dependence of dissociation constants for binding of L-phenylalanine (○) and L-phenylalaninol (●) to L-phenylalanine:tRNA ligase, and L-phenylalaninol to L-phenylalanine:tRNA ligase–MgATP complex (■). Experimental conditions are those described under Tables I and VIII.

temperature independent (parallel lines in Figure 2) indicating that synergism was entropy driven (approximately the same values for the reaction enthalpies in Table VIII).

Table VIII: Thermodynamic Parameters of the Formation of Binary and Ternary Complexes with L-Phenylalanine: tRNA Ligase.[a]

Reaction	Standard Reaction	
	Free Enthalpy (kcal)	Enthalpy (kcal)
Phe-ol + E ⇌ Phe-ol·E	−4.0	−8.3
Phe + E ⇌ Phe·E	−6.3	−7.5
Phe-ol + E·MgATP ⇌ Phe-ol·E·MgATP	−7.5	−7.0

[a] 25°, 0.01 *M* sodium–potassium phosphate buffer (pH 7.5); 0.1 m*M* EDTA, 0.1 m*M* dithiothreitol.

Table IX: The Effect of Spermine on the Activity of Amino Acid: tRNA Ligases in the tRNA-Aminoacylation Assay.[a]

Amino Acid: tRNA Ligase	Spermine (m*M*)	$MgCl_2$ (m*M*)	tRNA Aminoacylation	
			Cpm	Relative Activity
L-Phenylalanine		10	1050	1.0
	2.5	0.8	0	0.0
L-Isoleucine		10	410	1.0
	2.5	0.8	525	1.28

[a] The rate of tRNA aminoacylation was measured at 28°, pH 7.5. Conditions and procedures were the same as reported by Kosakowski and Böck (1970) except that magnesium had been omitted and that spermine had been included into the reaction mixture prior to addition of tRNA and enzyme. tRNA was unfractionated and had been equilibrated against 1 m*M* $MgCl_2$.

Discussion

The strength of coupling when ligands bind to enzymic sites normally recognized by amino acid and ATP ($MgATP^{2-}$) depends strongly, as expected, on the structural properties of these ligands. We may hypothesize that each of the structural components of these ligands interacts with a particular subsite of the substrate recognition site. Thus, $MgATP^{2-}$ may interact with a subsite for adenosine or AMP, for the triphosphate or pyrophosphate moiety and for the cation. The amino acid may bind to subsites accepting the side chain, the ammonium group, and the carboxylic group. For the L-isoleucine- and L-phenylalanine-specific enzymes of amino acid:tRNA ligases the existence of subsites has been considered already in detail (Santi et al., 1971; Holler et al., 1972, 1973; Holler, 1973; Mulivor and Rappaport, 1973; Flossdorf and Kula, 1973). Coupling of ligand binding may, therefore, be related to interaction of structural components with their subsites.

Cation Requirements. We have experienced that the association of the L-leucine and L-phenylalanine enzymes with the amino alcohols is impaired by the presence of magnesium. It appears as if these ligands were electrostatically repelled by the cation binding close to the active site. Uptake of magnesium by free L-phenylalanine:tRNA ligase has been reported (Holler et al., 1972; Hanke et al., 1974). In the case of amino acids, where the repulsion can be compensated by attraction between the cation and the carboxylic group, magnesium has no effect.

The affinity of ATP for complex formation was enhanced by the presence of magnesium for L-phenylalanine:tRNA ligase whereas it was decreased for L-leucine:tRNA ligase. A decrease has been recently reported for the L-methionine-specific enzyme (Fayat and Waller, 1974). Since ATP is known to form a complex with magnesium under our present conditions (Martell and Schwarzenbach, 1956), an interpretation of these findings would have to include either interactions of magnesium with the enzyme and ATP.

We have found in previous titration experiments that the binding of magnesium (and also of spermine) to the L-isoleucine- and L-phenylalanine-specific enzymes was accompanied by a strong enhancement of the fluorescence of the reporter group TNS (Holler, 1973; Holler et al., 1972). Since the dye was bound at, or close to, the active-site, it could have changed its fluorescence properties because of a rearrangement of the active site. In the particular case of L-phenylalanine:tRNA ligase a recent reactivation investigation with pH 2 dissociated enzyme has indicated that the three-dimensional structure of the active site might be under the control of the cation (Hanke et al., 1974).

The lack of effect of magnesium upon the binding of the amino alcohol or ATP to L-isoleucine:tRNA ligase is paralleled by its lack of effect on the synergistic binding of ligands. Similarly, synergistic coupling and tRNA aminoacylation are only moderately affected by spermine (Tables III and IX). In contrast, spermine abolishes coupling and aminoacylation for L-phenylalanine:tRNA ligase. The coincidence of the observations gives support to the idea that synergistic coupling is an indication of some rearrangement of the enzyme–substrate complex in an early step of the catalytic pathway.

The inhibition of the synergistic reaction by spermine most probably results from a replacement of magnesium by

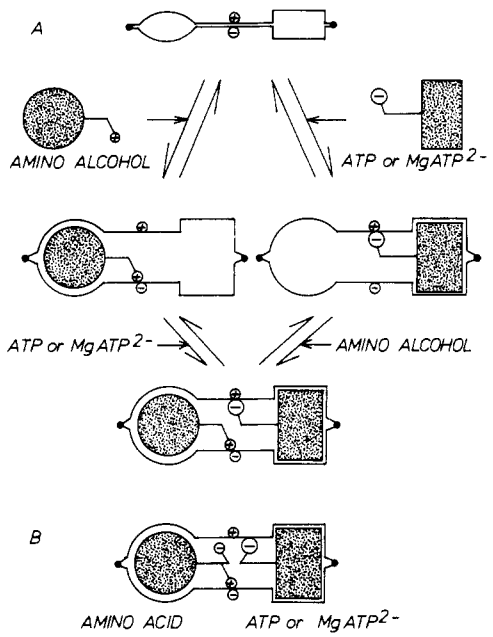


FIGURE 3: Schematic presentation of the synergistic coupling during formation of ternary amino acid:tRNA ligase–ligand complexes. (A) Intermolecular coupling between an amino alcohol and ATP or $MgATP^{2-}$, respectively. In the free enzyme, the access to the binding sites specific for amino acid and ATP is impaired by an interacting ion pair. Upon occupation of one of the sites, the other site is simultaneously opened up together with the ion pair. The second ligand can now enter readily stabilizing the ternary complex by interactions with fragments of the ion pair and the site adopting the side chain or the adenosine moiety, respectively. (B) Intramolecular synergism is visualized as a contribution to complex stability via interaction of the amino acid carboxylate with the cationic group of the former ion pair. Addition of ATP (or $MgATP^{2-}$) to the enzyme–amino acid complex is associated with an accumulation of negative charges and thus repulsion between carboxylate and triphosphate moieties. In this case, intermolecular synergism is impaired or completely cancelled.

spermine at the site controlling structural and binding properties of L-phenylalanine:tRNA ligase. Spermine is known to frequently replace magnesium in a variety of reactions (Bachrach, 1970; Stevens, 1970; Igarashi et al., 1971). The increased positive charge after replacement of the metal ion might then interfere with the electrostatic interactions of the model to be proposed in the following section.

Structural Requirements. The analysis of the correlation between coupling and ligand structure has been carried out for the L-phenylalanine-specific and L-isoleucine-specific enzymes. The two main results are the intramolecular synergism upon binding of the structural components of the amino acids and the intermolecular synergism for binding of the amino alcohols and ATP (or $MgATP^{2-}$), and the similarity of the strength of synergism pertaining to the intra- and intermolecular coupling. Upon inspection of Table I, one finds that these results are also obtained for the L-leucine-specific enzyme.

The structural component of central importance is undoubtly the side chain of the amino acid. It is involved in all the synergistic reactions whereas this is not the case for the adenosine moiety, the ammonium group, the carboxylic group, or the triphosphate moiety. This is an interesting finding which may be related to the high specificity found with these enzymes for the "correct" amino acid. A second requirement for synergism is the presence of an electrostatically charged group. Moreover, this group must carry the proper charge in order to achieve the strongest synergism. This is seen from a comparison of the strength of coupling for the binding of 2-methylbutylamine in the presence of AMP and ATP (Holler et al., 1973). Also, the charged group must be located in a proper position with respect to the geometry of the side chain. This is demonstrated by the decrease of the strength of coupling when the ammonium group of L-phenylalaninol is shifted toward the phenyl ring (e.g., with benzylamine, Table IV). Although we are aware of other mechanisms (like hydrogen bonding), which might explain the effects of the electrostatically charged groups, we favor the following working hypothesis. This hypothesis has been suggested previously on the basis of binding experiments with L-isoleucine:tRNA ligase (Holler et al., 1973). The details of the model are depicted in Figure 3. The active site or at least the binding sites for the amino acid and ATP (or $MgATP^{2-}$) are not easily accessible or not fully assembled in the free enzyme because of an interacting ion pair. The binding of either ligand causes the simultaneous opening of the ion pair. This requires energy. Once the ion pair is opened, further energy is not needed, and a second ligand binds more readily than if it had been first.

The model fulfills the requirements which have been worked out on the basis of the experimental results. In particular, the model predicts electrostatic interaction with the ionic structural components of the amino acid and ATP. In either the deamino acid and the decarboxy amino acid (the ammonium group and the carboxylic group, respectively, are removed) the charged group is attracted by the oppositely charged group of the ion pair, but simultaneously repelled by the equally charged group of the ion pair. Both effects may cancel leading to a similar dissociation constant for the binding of these ionic ligands and the corresponding alcohols. In the zwitterionic amino acid the components of the ion pair can interact with two oppositely charged groups of the ligand leading to stabilization of the complex. Similar to this mechanism of intramolecular synergism is that for the intermolecular synergism. Here, the interaction of the carboxylate group with the positively charged group of the ion pair is replaced by the interaction between this group and the triphosphate moiety. When the ionic component of a ligand is moved out of its optimal position as is the ammonium group of benzylamine, then the electrostatic interaction with one component of the ion pair is weakened because of the distance. When the two ligands are the amino acid and ATP ($MgATP^{2-}$), the opposition of the carboxylate group and the triphosphate moiety (± magnesium) causes repulsion and antagonism rather than synergism (see also dehydrocinnamic acid in Table V). The same conclusion was obtained recently from the synergistic binding of L-phenylalanine and the various adenosine nucleotides (Kosakowski and Holler, 1973).

We have mentioned the electrostatic part of the model. In addition, there must be another part which enhances the binding of L-phenylalaninol in the presence of adenosine, or the binding of 3-methyl-1-pentanol in the presence of ATP (Tables VI and VII). Apparently, the opening of the ion pair together with the binding of adenosine to its subsite could induce a proper assembly of the side-chain subsite to achieve a tighter binding.

Since the opening of the ion pair might involve relatively slow rearrangements of amino acid moieties at the active site, this process could be related to the slow, secondary process which was observed recently for the rapid binding kinetics of L-isoleucine to L-isoleucine:tRNA ligase (Holler and Calvin, 1972). It was also found that this slow process

induced the fluorescence change of the reporter group, TNS, which is bound closely to the active site (Holler et al., 1971).

Despite the observed consistency it must be emphasized that the ion-pair model serves only as a working hypothesis. Further investigations are necessary to establish its validity.

The function of the synergistic process is not known. It may be indicative of an early step during the enzymic reaction. As we have argued earlier (Kosakowski and Holler, 1973), the synergism might be used by the enzyme to compensate for the repulsion between the negative charges of the triphosphate moiety and the carboxylic group during formation of the aminoacyl adenylate. Such an input of free energy upon formation of the ternary enzyme–substrate complex would explain the more favorable formation of the enzyme-bound aminoacyl adenylate from enzyme-bound reactants in comparison with its formation from free reactants in solution (Holler and Calvin, 1972).

The L-isoleucine, L-leucine, and L-phenylalanine:tRNA ligases have different quaternary structures (Kisselev and Favorova, 1974). The isoleucyl- and leucyl-enzymes are single polypeptides while the L-phenylalanyl-enzyme is of the $\alpha_2\beta_2$ type (Hanke et al., 1974). Nevertheless, synergism is observed to be independent of the structure. Moreover, recent evidence has been accumulated that the L-phenylalanyl enzyme has two, however, not cooperatively functioning, active sites (P. Bartmann, 1975, submitted for publication). The L-isoleucyl- and L-leucyl-enzymes have single sites. It is, therefore, unlikely that synergism involves binding of a first and second ligand to different active sites. The similar results obtained from our structural analysis provide evidence that the mechanism may be similar for the three enzymes despite their gross structural differences. Whether the synergistic reaction and, in particularly, the proposed mechanism will be valid for other synthetases remains to be investigated in detail.

That synergistic binding between ATP or structural components thereof and second ligands is not restricted to amino acid:tRNA ligases (Kosakowski and Holler, 1973) is evident from observations with other enzyme systems, such as firefly luciferase (Bowie et al., 1973), xanthosine 5′-phosphate aminase (Zyk et al., 1969), or glutamine synthetase (Timmons et al., 1974).

Acknowledgments

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