On the Active Site Topography of Isoleucyl Transfer Ribonucleic Acid Synthetase of *Escherichia coli* B†

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ABSTRACT: The topography of the active site of Ile-tRNA synthetase has been investigated by using structural analogs for L-isoleucine and ATP. Radioactive ATP-[^{32}P]PP$_i$ exchange methods and fluorescence titration methods were applied for determination of binding properties of substrates and inhibitors. The results are consistent with a hydrophobic region at the L-isoleucine specific site to accommodate the aliphatic side chain. The α-amino group is electrostatically bound to an anionic site. It is believed that this site is part of an ion pair in the free enzyme. The ion pair can be opened by either L-isoleucine and related compounds or by ATP, AMP, and pyrophosphate. Opening is coupled to the interaction of the hydrophobic side chain. When the electrostatic interaction is sterically prevented, as in case of methylation or guanidation of the α-amino group, hydrophobic interaction is also prohibited. The ion pair can be easily opened by the intact substrate, when both the α-amino group and carboxylic group are present. Compounds lacking the carboxylic group are good ligands only when ATP or AMP is simultaneously bound to the enzyme. This observation has an important bearing on the use of α-amino alcohols as inhibitors for elucidation of the catalytic pathway. Free-energy considerations indicate that the affinity for binding of ATP to Ile-tRNA synthetase is 4 kcal less than anticipated from the binding affinities measured for its structural components, AMP and pyrophosphate. Furthermore, the standard free energy of formation of enzyme-bound L-isoleucine adenylate and pyrophosphate from enzyme-bound substrates is favored by 5 kcal as compared with the reaction in the free solute state. We conclude that ATP when enzymically bound is in a high free-energy state, thus facilitating the formation of enzyme-bound L-isoleucine adenylate.

Probing of the active site of enzymes has been accomplished by binding experiments using a structural variety of substrates and inhibitors as, for instance, in the case of chymotrypsin (Blow,[1] 1971) and trypsin (Keil,[1] 1971). This method has not been rigorously applied to the family of aminoacyl-tRNA synthetases except for its recent use on Phe-tRNA synthetase (Santi *et al.*, 1971), on Val-tRNA synthetase (Owens and Bell, 1968), and on Pro-tRNA synthetase (Papas and Mehler, 1970).

In this article we report results obtained for probing of the active site of Ile-tRNA synthetase using compounds structurally related to L-isoleucine, ATP, and pyrophosphate, which are the substrates for the amino acid activation reaction described by the following, simplified equation.[2]

$$E + ATP + Ile \underset{}{\overset{(Mg^{2+})}{\rightleftharpoons}} E \cdot Ile{\sim}AMP + PP_i$$
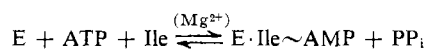
It is believed that the enzyme-bound intermediate, L-isoleucyl adenylate, reacts in a second step with the cognate tRNA^{Ile} to form Ile-tRNA^{Ile} (Berg, 1958) according to the following equation.

$$E \cdot Ile{\sim}AMP + tRNA^{Ile} \rightleftharpoons Ile\text{-}tRNA^{Ile} + E + AMP$$

Possibly, formation of the Ile-tRNA synthetase–tRNA^{Ile} complex is associated with some rearrangement at the active site. With this in mind, we must distinguish between probing of the active site of free enzyme and probing of tRNA^{Ile}-bound enzyme. From comparison of the results the extent and the consequences of the tRNA^{Ile}-induced rearrangement will be evident.

The investigation reported in this paper is restricted to the probing of the free enzyme only. L-Isoleucine related compounds lacking either the α-amino group or the carboxylic group, or both, were used as probes. Further, the effects of methylation and guanidation of the α-amino group and the effect of chain elongation of the L-isoleucyl side chain on binding properties, catalytic properties, and fluorescence properties were evaluated. Similarly, we have measured the extent of binding of the structural components of ATP, *i.e.*, adenine, D-ribose, adenosine, AMP, and pyrophosphate. In part, we have examined the interactions of inhibitors in the presence of co-ligands. Part of this investigation has been published recently (Holler *et al.*, 1971).

Materials and Methods

Ile-tRNA synthetase was obtained as a 300- to 350-fold purified preparation from *Escherichia coli* B cells (Miles Laboratory) following a method described by Baldwin and Berg (1966). Protein was determined by the method of Lowry *et al.*, (1951). The specific activity of the freshly purified enzyme was 650–750 units/mg, where one unit is defined as the formation of 1 μmol of [^{32}P]ATP from [^{32}P]PP$_i$ in 15 min at 37° under standard conditions (Baldwin and Berg, 1966). The published value of the molecular weight (112,000, Baldwin and Berg, 1966) was used to calculate the enzyme

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[1] Reference is made to review articles.

[2] Abbreviations used are: E, isoleucyl transfer ribonucleic acid (Ile-tRNA) synthetase; E·Ile·ATP, Ile-tRNA synthetase–L-isoleucine–adenosine triphosphate complex; Ile$\sim$AMP, L-isoleucyl adenylate; E·Ile$\sim$AMP·PP$_i$, Ile-tRNA synthetase–L-isoleucyl adenylate–pyrophosphate complex.

concentration. Enzyme was stored at −15° in 0.02 M sodium phosphate buffer, pH 7.5, which contained 0.1 mM glutathione.

tRNA was purified from *E. coli* B cells (Schwarz BioResearch) according to the derivation method described by Gilliam *et al.* (1967, 1968). Finally, the preparation was purified over a Bio-Gel P20 column (57 cm × 1 cm) by elution with 0.02 M phosphate buffer, pH 8.0, at 4°. The preparation was 20% pure tRNA^{Ile} (in terms of A_{260} units). No distinction has been made between different isoaccepting forms of tRNA^{Ile}. The concentration of total tRNA was estimated spectrophotometrically on the assumption that 21.4 A_{260} units are equivalent to 1 mg/ml of tRNA (Stephenson and Zamecnik, 1961).

[^{32}P]PP$_i$ was prepared from [^{32}P]P$_i$ (International Chemical and Nuclear Corp.) as described by Berg (1958). Guanidino-L-isoleucine was prepared from L-isoleucine and 5-methylthioisourea following the method described by Fasold *et al.* (1961) and from L-isoleucine and 1-guanyl-3,5-dimethylpyrazole nitrate as described by Habeeb (1960). Both preparations gave identical results; the material was negative against ninhydrin, soluble in water, and melted at 228° with decomposition (the closely related compounds for leucine and valine melt with decomposition at 242 and 240°, respectively, Prager and Jacobson, 1944). 3-Methylpentanoic acid, 3-methyl-1-pentanol, and 2-methyl-1-butanol were freshly redistilled before use. Amino acids and amines were found to be homogeneous by thin-layer chromatography (Eastman Chromagram, 6060 Silica Gel). Solvent mixtures were *n*-butyl alcohol saturated with 20% acetic acid, and benzene-pyridine-acetic acid (80:20:5, v/v/v).

The L-isoleucine ethyl and hexyl esters were prepared as the hydrochlorides with thionyl chloride in the appropriate alcohol by the method of Brenner and Huber (1953) with minor modifications. No attempt was made to distill the esters. L-Isoleucine ethyl ester hydrochloride had a mp of 92–93°. *Anal.* Calcd for $C_8H_{18}ClNO_2$: C, 49.09; H, 9.27; Cl, 18.14; N, 7.15. Found: C, 49.23; H, 9.25; Cl, 18.20; N, 7.36.

The hexyl ester hydrochloride could not be crystallized and was obtained as an amorphous, waxy solid. *Anal.* Calcd for $C_{12}H_{26}ClNO_2$: C, 57.20; H, 10.40; Cl, 14.10; N, 5.60. Found: C, 57.0; H, 10.5; Cl, 13.9; N, 5.5.

Both compounds were homogeneous to thin-layer chromatography on silica gel using 7:3 acetone-water or 9:1 chloroform-triethylamine.

L-Isoleucinyl adenylate was synthesized by the procedure of Sandrin and Boissonnas (1966) and Cassio *et al.* (1967) with minor modifications. The product was homogeneous to thin-layer chromatography on silica gel using methanol or 7:3 acetone-water: ultraviolet (uv) (0.01 M potassium phosphate, pH 7.5) max 259 nm (ε 12,100); min 227 nm. *Anal.* Calcd for $C_{16}H_{27}N_6O_7P \cdot 0.3H_2O$: C, 42.5; H, 6.2; N, 18.6; P, 6.9. Found: C, 42.6; H, 6.2; N, 18.5; P, 6.9.

3-Methyl-1-pentanol and 3-methylpentanal were obtained from Aldrich, 2-methyl-1-butylamine and 3-methylpentanoic acid from K&K Laboratories, and 2-methyl-1-butanol, α-DL-aminopentanoic acid, α-DL-aminohexanoic acid, and α-DL-aminoheptanoic acid from Eastman Organic Chemicals. O-Methyl-L-threonine, N-methyl-DL-isoleucine, valine, adenosine, and AMP were purchased from Calbiochem, L-isoleucine methyl ester, potassium 2-*p*-toluidinylnaphthalene-6-sulfonate, and S-methylisothiourea sulfate from Sigma, L-isoleucine, L-isoleucinol, and ATP from Nutritional Biochemical Corp., and 1-guanyl-3,5-dimethylpyrazole and adenine from Schwarz/Mann. All other chemicals were reagent grade and purchased from Baker Chemical Co. Deionized and distilled water was used which had been boiled and cooled under nitrogen to remove oxygen and carbon dioxide.

Radioactive ATP-[^{32}P]PP$_i$ Exchange Reaction. The method used was essentially the method described by Baldwin and Berg (1966), at pH 8.0, 25°, in 0.05 M Tris-HCl buffer and 0.01 M 2-mercaptoethanol. Radioactivity was measured as counted by a Nuclear-Chicago end-window Geiger counter. The amino acid specific exchange reaction was measured as a function of varying concentrations of amino acid. Numbers of counts per minute were plotted as a function of concentration of substrate according to the method of Eadie (1942) or Lineweaver and Burk (1934). The value for the Michaelis–Menten constant, $K_m(app)$, and its value in the presence of an inhibitor at a fixed concentration, $K_m^I(app)$, were determined from the slope of the linear plots. The inhibition constant for competitive inhibition was calculated according to $K_i = [I]_0/[(K_m^I(app)/K_m(app)) - 1]$ where the symbol $[I]_0$ refers to the initial concentration of the inhibitor. For α-DL-aminohexanoic acid and α-DL-aminoheptanoic acid, the Michaelis–Menten constant was evaluated from a set of data where the concentration of a competitive inhibitor, 2-methyl-1-butylamine, was varied and the concentration of the substrate was kept constant. Evaluation is accomplished by use of the equation

$$v = v_0 - v[I]_0 K_m(app)/[K_i(K_m(app) + [S]_0)] \qquad (1)$$

which is derived as follows. The Michaelis–Menten equations for the absence and presence of a competitive inhibitor are

$$v_0 = V_{max}\frac{[S]_0}{K_m(app) + [S]_0}$$

and

$$v = V_{max}\frac{[S]_0}{K_m(app)[1 + [I]_0/K_i] + [S]_0}$$

respectively. The symbols refer to v_0, the rate in the absence of inhibitor; v, the rate in the presence of inhibitor; V_{max}, the maximum rate; $[S]_0$ and $[I]_0$, initial concentration of substrate and inhibitor, respectively; $K_m(app)$ and K_i, the apparent Michaelis–Menten constant and inhibition constant, respectively. Dividing the first equation by the second and rearranging, eq 1 is obtained. The data in Figure 1 were plotted according to this equation and the value for $K_m(app)$ was calculated from the slope using the value $K_i = 10$ μM. The procedure is generally applicable as long as substrate and inhibitor are in excess of enzyme. The same procedure has been previously described by Inagami (1964). Maximum exchange rates for these two analogs were determined from the Michaelis–Menten equation, using the rate of exchange as measured in the absence of the inhibitor. Initial concentrations of the reaction mixtures were 0.5–1.5 nM Ile-tRNA synthetase, 2 mM ATP, 2 mM sodium pyrophosphate (4 × 10⁴–8 × 10⁴ counts min⁻¹ μmol⁻¹), 5 mM MgCl₂, 10 mM KF, 0.1 M Tris-HCl, 10 mM 2-mercaptoethanol, 3 μM to 0.6 mM L-isoleucine, or 3 μM to 0.6 mM O-methyl-L-threonine, or up to 1.2 mM N-methyl-DL-isoleucine or guanidino-L-isoleucine, or up to 27 mM α-DL-aminopentanoic acid, or up to 29.8 mM α-DL-aminohexanoic acid, or up to 6.9 mM α-DL-aminoheptanoic acid. Inhibitors were used at concentrations higher
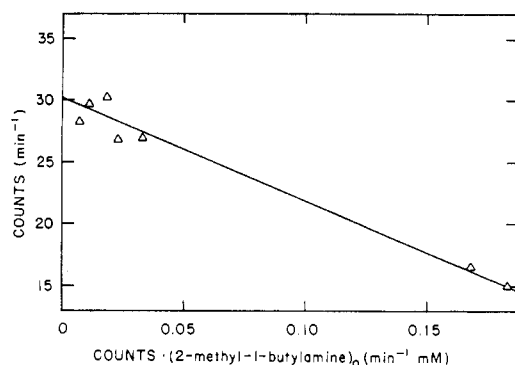
FIGURE 1: Determination of the Michaelis–Menten constant for ATP-[^{32}P]PP$_i$ exchange maintained in the presence of α-DL-aminoheptanoic acid, at pH 8.0, 25°. The rate of exchange is measured as a function of various concentrations of 2-methyl-1-butylamine, a competitive inhibitor. Evaluation is based on the equation $v = v_0 - v[I]_0 K_m(\text{app})/[K_i(K_m(\text{app}) + (S)_0]$, where the symbols refer to v_0, the rate of exchange when no inhibitor is present, $[I]_0$ and $[S]_0$, the initial concentrations of inhibitor and substrate, respectively. Initial concentrations were 5 nM Ile-tRNA synthetase, 3.45 mM α-DL-aminoheptanoic acid, 2 mM ATP, 2 mM sodium pyrophosphate (6 × 10^4 cpm μmol^{-1}), 5 mM MgCl$_2$, 10 mM KF, 0.1 M Tris-HCl, and 0.01 M 2-mercaptoethanol.

than indicated by the inhibition constant. 2-Methyl-1-butylamine was varied from 0.12 to 12.3 μM in the presence of α-DL-aminohexanoic acid at 29.8 mM or α-DL-aminoheptanoic acid at 3.45 mM. Exchange reactions for ATP and related compounds were conducted at 37°, pH 8.0. Initial concentrations of the reaction mixtures were 12 nM Ile-tRNA synthetase, 2 mM L-isoleucine, 2 mM sodium pyrophosphate [1 × 10^4– 2 × 10^4 counts min^{-1} μmol^{-1}), 10 mM KF, 10 mM 2-mercaptoethanol, 0.1 M Tris-HCl, and 0.05-5 mM ATP. The MgCl$_2$ concentration was varied concomitantly with ATP to provide a 1 mM excess of MgCl$_2$ over the total concentration of ATP plus pyrophosphate. Inhibitor concentrations were: AMP, 2 mM; adenosine, 5.5 mM; and adenine, 10 mM.

tRNA-Charging Reaction. Potassium 2-p-toluidinylnaphthalene-6-sulfonate (TNS) was tested for inhibition of the tRNA charging reaction using a procedure similar to that described by Muench and Berg (1966).

The amount of incubation mixture used was 800 μl. Initial concentrations were 1 mM ATP, 3 mM MgCl$_2$, 0.025 mM L-[^{14}C]isoleucine (specific activity 25 μCi/μmol), 0.2 mM TNS, 0.04-0.4 260-nm absorbance units of tRNA, 0.4 M Tris-HCl buffer, 1 mM 2-mercaptoethanol, 5 mM KCl, and 0.005 μM Ile-tRNA synthetase. TNS was omitted in control experiments. The mixture was incubated at 24° and 100-μl samples were withdrawn at 5-min intervals. The samples were immediately mixed with 200 μl of an ice-cold solution of 20 mg/ml of RNA from Torula (Calbiochem, lot 44585) and precipitated upon addition of 2 ml of 2 N HCl. The precipitate was allowed to stand on ice for 5 min and was then filtered through a glass fiber filter (Whatman GF/C). The filter was washed several times with 2 N HCl and 45% ethanol, dried, and finally counted in a Packard Tri-Carb scintillation counter.

Fluorescence Titration. The amount of complex formation of a ligand with Ile-tRNA synthetase was measured as the degree of quenching of the fluorescence of TNS which was reversibly bound to the enzyme. The method has been previously described (Holler *et al.*, 1971; Holler and Calvin, 1972). Fluorescence was measured with a Perkin-Elmer

fluorescence spectrophotometer, Model MPF-2A. Excitation at 290 nm of the enzyme tryptophan residues as well as excitation at 366 nm of TNS and emission at 470 nm were used. As described previously (Holler *et al.*, 1971), the degree of quenching was higher when the 290-nm excitation was used. Slits were 10 nm for both excitation and emission light path. The sample compartment was thermostated within ±0.5° and was flushed with nitrogen.

In a typical titration experiment aliquots of 0.5 or 1 μl of a solution containing the ligand under study were added from a syringe to 700 μl of buffer containing enzyme and TNS. The mixture was carefully stirred with the needle and 3 min were allowed for the temperature to equilibrate. Radiation was admitted only for the period of fluorescence measurement (about 3 sec) in order to prevent possible photodecomposition. Titration curves were corrected for dilution as measured separately using distilled water instead of titrant. Concentrations of enzyme and TNS were 0.05-0.15 μM, and 5-20 μM, respectively. Buffers were 0.05 M Tris-HCl and 0.02 M sodium phosphate; all solutions contained 0.01 M 2-mercaptoethanol unless otherwise stated.

Under all conditions except for L-isoleucinyl adenylate, ligand was in excess of enzyme and the linearization procedure described by Eadie (1942) was used for evaluation of the dissociation constant and of the maximum quenching at (extrapolated) infinite concentration of ligand. The concentration of L-isoleucinyl adenylate was comparable with the concentration of enzyme and the degree of fluorescence quenching was evaluated according to the method of Dahlquist *et al.* (1966).

Titrations were mostly done at pH 8.0; when pH was varied, sufficient NaCl was added to provide a constant ionic strength of 0.05 M. Errors were calculated as mean deviations for two or more determinations.

Kinetic Measurement. The kinetics of binding of L-isoleucine and 2-methylbutylamine to Ile-tRNA synthetase were measured on a Durrum-Gibson stopped-flow spectrophotometer which had been modified so as to observe the TNS fluorescence as previously described (Holler and Calvin, 1972). Reactions were initiated by rapid mixing of a solution containing enzyme and TNS with an equal volume of a solution containing TNS and the ligands under study. Solutions were made in 0.05 M Tris-HCl buffer, pH 8.0. An excitation wavelength of 290 nm was used and emission was observed through a cut-off filter, Corning No. 373 (35% transmission at 416– 436 nm and 80% at 511 nm). The rate constants of the observed reactions were determined from the slopes of the first-order plots and evaluated on the basis of an apparent bimolecular reaction mechanism (Holler and Calvin, 1972) according to the expression

$$k_{\text{obsd}} = k_{21} + k_{12}[\text{ligand}]_0 \qquad (2)$$

The symbols k_{12} and k_{21} refer to the rate constants for the formation and dissociation, respectively, of enzyme–ligand complexes. The subscript zero indicates initial concentrations ($\gg$[E]$_0$).

Results

Effect of TNS on the tRNA Charging Reaction. Figure 2 shows the degree of charging as function of time for three different initial concentrations of tRNA. The solution was saturated with TNS which gave a sufficient concentration to complex most of the enzyme ($K_s = 0.07$ mM; Holler *et al.*,
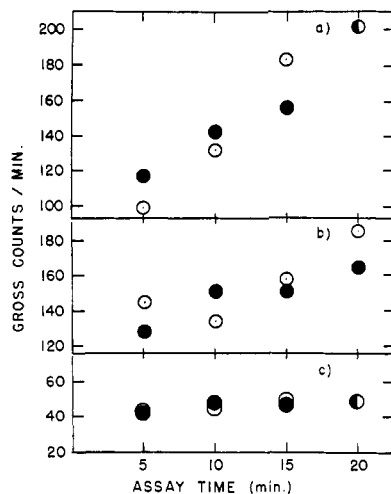
FIGURE 2: tRNA^{Ile}-charging reaction catalyzed by Ile-tRNA synthetase in the presence of TNS at pH 8.0, 25°. The extent of amino acylation of tRNA^{Ile} was measured as a function of time in the presence of 0.2 mM TNS (●) and in the absence of TNS (○). Initial concentrations were 1 mM ATP, 3 mM MgCl$_2$, 0.025 mM L-isoleucine, 5 nM Ile-tRNA synthetase, 0.4 M Tris-HCl buffer, 1 mM 2-mercaptoethanol, 5 mM KCl and (a) 0.15 μM, (b) 0.09 μM, and (c) 0.02 μM tRNA^{Ile}. Activities are not corrected for background activities and losses due to adsorption effects. It is seen that the extent of charging as a function of time is the same, within experimental error, whether or not TNS is added to the reaction mixture.

FIGURE 3: ATP-[^{32}P]PP$_i$ exchange for L-isoleucine (○) and O-methyl-L-threonine (●), at pH 8.0, 25°. Plot in the inset according to linearization procedure of Eadie (1942) for evaluation of maximum exchange rate (from intercept) and Michaelis–Menten constant (from slope). The broken line has been calculated from parameters, which have been determined from a separate Eadie plot for L-isoleucine. Exchange is not observed for N-methyl-DL-isoleucine (bottom). Initial concentrations were 1 nM Ile-tRNA synthetase, 2 mM ATP, 2 mM sodium pyrophosphate (8 × 10^4 cpm μmol^{-1}), 5 mM MgCl$_2$, 10 mM KF, 0.1 Tris-HCl, and 0.01 M 2-mercaptoethanol.

1971). tRNA^{Ile} and L-isoleucine were at concentrations close to their dissociation constants, 0.2 μM (Baldwin and Berg, 1966) and 5 μM (Cole and Schimmel, 1970; Holler et al., 1971), respectively, so as to provide sensitivity against TNS-induced changes of kinetic and equilibrium parameters. Data are not corrected for background activities. Rates were expected to be inexact because of adsorption effects at the low concentrations of reactants applied (Demushkin et al., 1971). However, if TNS does not impair the enzyme activity, we should find the same activities in the absence and presence of the dye. According to Figure 2, there is agreement within the experimental error suggesting that TNS, at 0.2 mM, has little or no effect on the enzyme activity. The same conclusion has been reached with respect to the amino acid activation reaction (Holler et al., 1971).

L-*Isoleucine and Related Compounds.* The interaction between Ile-tRNA synthetase and various compounds which are structurally related to L-isoleucine was investigated in terms of Michaelis–Menten constants or inhibition constants and maximum ATP-[^{32}P]PP$_i$ exchange rates and in terms of dissociation constants and maximum quenching of fluorescence intensity as measured in the titration experiments. The results are listed in Table I. The kinetic parameters of the exchange reactions were evaluated directly from Eadie plots (Eadie, 1942) except for α-DL-aminohexanoic acid and α-DL-aminoheptanoic acid. Since the level of exchange for these was low we found it easier to determine the values for K_m(app) from inhibition experiments, as described under Materials and Methods.

We have determined the type of inhibition with respect to L-isoleucine for 2-methyl-1-butylamine, α-DL-aminohexanoic acid, α-DL-aminoheptanoic acid, and O-methyl-L-threonine. The inhibition was found to be competitive.

Finally, we have determined the values for the maximum rate of exchange and for the maximum quenching of the fluorescence. The values listed in Table I refer to those for
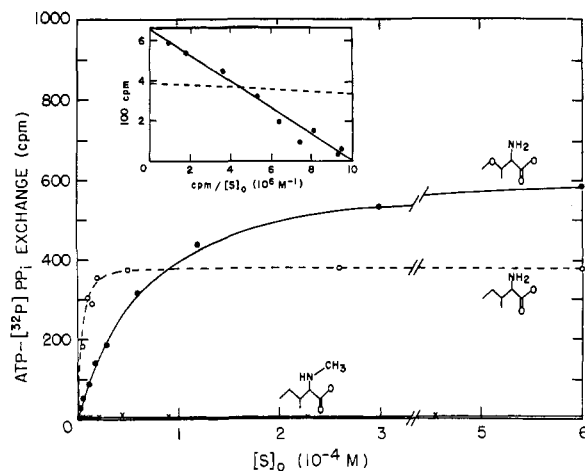
L-isoleucine as unity. Experimental errors determined as standard deviations are between 10 and 15% of the values listed. Maximum quenching as compared to fluorescence in the absence of substrate or inhibitors was about 40% for L-isoleucine, ATP, or pyrophosphate (Holler et al., 1971).

From Table I the following observations were made. (1) in addition to L-isoleucine, radioactive exchange was maintained in the presence of O-methyl-L-threonine (Figure 3), L-valine, α-DL-aminopentanoic acid, α-DL-aminohexanoic acid, and α-DL-aminoheptanoic acid. The rate of exchange decreased in the order of listing. O-Methyl-L-threonine had a 50% higher exchange rate than L-isoleucine. (2) There is no obvious correlation between the values of the maximum rate of ATP-[^{32}P]PP$_i$ exchange and the value for the Michaelis–Menten constant. The apparent decrease of the maximum rate for the racemic substrates is presumably due to inhibition by the unreactive D enantiomer (Mehler, 1970). Similarly, we found that the degree of maximum quenching of the fluorescence was not a function of the dissociation constant, but was the same, within experimental error, for all substrates tested. (3) Values for K_m(app) and K_s are almost identical, indicating that binding of co-ligands (as in the ATP-[^{32}P]PP$_i$ exchange) had little, if any, effect on the dissociation constant as measured by fluorescence titration. (4) Modification of the amino group of L-isoleucine by either methylation or guanidation was associated with the loss of binding. (5) When the 3-methyl group or the terminal methyl group was removed from the side chain, the stability of the enzyme–amino acid complex was considerably reduced. Elongation as measured for α-DL-aminohexanoic acid and α-DL-aminoheptanoic acid had little effect on the maximum rate of exchange and only a modest effect on the value of the Michaelis–Menten constant. (6) Blocking of the carboxylic group as in L-isoleucine methyl, ethyl, and hexyl ester reduced the affinity for complex formation and, of course, abolished the catalytic reaction. Interestingly, the affinity increased as the length of the alcohol component increased. A similar observation has been reported by Santi et al.

TABLE I: Interaction of L-Isoleucine and Related Compounds with Ile-tRNA Synthetase at pH 8.0.

Compound[a]	Method[b]	$K_i{}^c$ (μM)	$K_m{}^d$ (μM)	$K_s{}^e$ (μM)	Max Quenching or Max Exchange Rate[f]
L-Isoleucine	F			5.8 ± 0.8	1.0[l]
	P			4.0[g]	
	P		5[h]		1.0
O-Methyl-L-threonine	F			(1.2 ± 0.2) × 10²	1.0
	P		60 ± 10		1.6
L-Valine	F			(1.6 ± 0.5) × 10²	
	P		3.9 × 10² [h]		~0.5
	P		8 × 10² [i]		0.8
α-DL-Aminopentanoic acid	F			(2.2 ± 0.7) × 10³	1.0
	P		(1.15 ± 0.1) × 10³		0.7
α-L-Aminopentanoic acid	P		1.5 × 10³ [i]		0.9
α-DL-Aminohexanoic acid	F			(7 ± 2) × 10³	1.0
	P		7 × 10³ [j]		0.35
α-DL-Aminoheptanoic acid	P		1.8 × 10⁴ [j]		0.35
N-Methyl-DL-isoleucine	F				None
	P	>10⁴			
Guanidino-L-isoleucine	F				None
	P	>10⁵			
L-Isoleucine methyl ester	F			(5.9 ± 0.8) × 10²	1.0
L-Isoleucine ethyl ester	P (37°)	2.7 × 10² [j]			
L-Isoleucine n-hexyl ester	P (37°)	40[j]			
3-Methylpentanoic acid	F			>1 × 10⁵	None
	P	>5 × 10⁵			
L-Isoleucinol	F (10°)			(5.5 ± 1) × 10³	1.0 (excitn 290 nm)
1.5 mM PP_i + 2 mM MgCl₂	F (10°)			~2 × 10³	
1 mM PP_i + 2 mM MgCl₂	F			(1.4 × 0.3) × 10³	1.0 (excitn 290 nm)
	P	23[k]			
L-Isoleucinyl adenylate	F (10°)			(1.3 ± 0.3) × 10⁻²	
	P	7 × 10⁻³ [k]			
3-Methyl-1-pentanol	F			(2.5 ± 1) × 10³	(excitn 290 nm)
	F (10°)			(1.6 ± 0.3) × 10³	1.0
3-Methylpentanal	F (10°)			(2.5 ± 1) × 10³	
2-Methyl-1-butanol	F			(1.7 ± 0.3) × 10⁴	1.0
	P	>5 × 10⁵			
2-Methyl-1-butylamine	F			(9 ± 1) × 10³	1.0
	F (12°)			(5.1 ± 1.0) × 10³	1.0
	P	10 ± 2			
2 mM PP_i	F			(9 ± 2) × 10²	
1 mM PP_i + 1.5 mM MgCl₂	F			(1.7 ± 0.6) × 10³	
1 mM ATP	F			3.8 ± 1.0	
1 mM ATP + 2 mM MgCl₂	F			4.5 ± 1.0	
1 mM ATP, 1 mM PP_i + 1.5 mM MgCl₂	F			2.5 ± 0.5	
4.6 mM AMP	F			27 ± 2	
1 mM ATP	Kinetically			2.6[j]	

[a] Investigated at 25° unless otherwise stated. [b] Method of investigation: P, ATP-[³²P]PP_i exchange; F, fluorimetric titration. Excitation wavelength 366 nm, emission wavelength 470 nm. [c] Inhibition constant from ATP-[³²P]PP_i exchange measurements. [d] Michaelis–Menten constant from ATP-[³²P]PP_i exchange measurements. [e] Dissociation constant from titration. [f] With reference to values for L-isoleucine, which were arbitrarily set equal to 1.0. [g] Cole and Schimmel, 1970. [h] Berg *et al.*, 1961. [i] Loftfield and Eigner, 1966. [j] Experimental error was not calculated. [k] Cassio *et al.*, 1967. [l] Standard deviations are 10–15%.

(1971) for Phe-tRNA synthetase from *E. coli*. (7) When the α-amino group was removed, as in 3-methylpentanoic acid, an inert compound was obtained. (8) Removal of the α-amino group and substitution of the carboxylic group by an alcohol or an aldehyde group, as in 3-methyl-1-pentanol or in 3-methylpentanal, reduced binding considerably but did not abolish it. (9) When the α-amino group was not removed and the carboxylic group was substituted by hydrogen or an alcohol group as in 2-methyl-1-butylamine and L-isoleucinol, respectively, the modified compounds were potent inhibitors in the radioactive exchange reaction but poor ligands for the fluorimetric titration in absence of co-ligands.
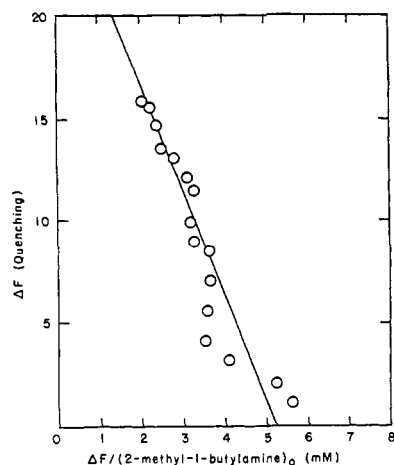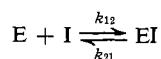
FIGURE 4: Titration of Ile-tRNA synthetase–TNS with 2-methyl-1-butylamine at pH 8.0, 12°. All other ligands were omitted from the reaction mixture. Initial concentrations were 0.19 μM enzyme, 12 μM TNS, 0.05 M Tris-HCl buffer, and 0.01 M 2-mercaptoethanol. Concentration of 2-methyl-1-butylamine was varied between 0.19 and 7.7 mM, excitation wavelength, 366 nm; emission wavelength, 470 nm. Light scatter was reduced by placing a 430-nm cutoff filter in the emission path.
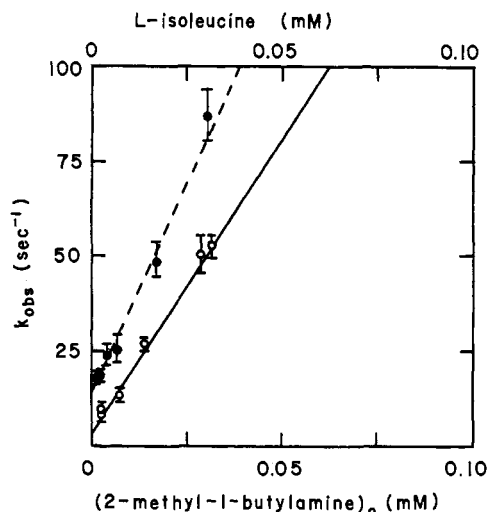

FIGURE 5: Kinetics of fluorescence quenching following rapid mixing of a solution containing enzyme with a solution containing ATP plus 2-methyl-1-butylamine (open circles) or L-isoleucine (filled circles), at pH 8.0, 25°. Experiments were accomplished with a modified Durrum-Gibson stopped-flow spectrofluorimeter. Initial concentrations were 0.075 μM Ile-tRNA synthetase, 43 μM TNS, 1 mM ATP, 0.05 M Tris-HCl, and 0.01 M 2-mercaptoethanol.

Binding of 2-Methyl-1-butylamine under Various Conditions. In the absence of co-ligands, 2-methyl-1-butylamine interacts only weakly with Ile-tRNA synthetase as measured by titration, Figure 4. Addition of pyrophosphate, AMP, or ATP at saturating concentrations was associated with a strong decrease of the value for the dissociation constant. ATP produced a decrease about 1000 times greater than that produced by pyrophosphate. The coupling was not sensitive to magnesium ions (<2 mM). In the presence of ATP or Mg-ATP^{2-} 2-methyl-1-butylamine binds almost as strongly as L-isoleucine, as evidenced from titration experiments. The value for the inhibition constant from the ATP–[^{32}P]PP$_i$ exchange reaction was found to be higher than expected for ATP as the only co-ligand. Our interpretation of this is that part of the enzyme is bound to pyrophosphate instead of to ATP. Since 2-methyl-1-butylamine binds more weakly when pyrophosphate is the co-ligand, the inhibition constant of the exchange reaction would indeed be expected to have a higher value.

Kinetics of Binding of 2-Methyl-1-butylamine in the Presence of ATP. The value of the dissociation constant for binding of 2-methyl-1-butylamine to Ile-tRNA synthetase–ATP is comparable with the value for L-isoleucine. It was of interest to see whether the kinetics were also similar. Fluorescence was measured as a function of time following the rapid mixing of a solution containing enzyme plus TNS with a solution containing ATP, TNS, and 2-methyl-1-butylamine at various concentrations. Only one process was observed which did not appear when the solutions containing the reactants separately were mixed with an enzyme solution. The observed rate constants were plotted as shown in Figure 5 as a function of the concentration. The linear concentration dependence is consistent with the apparent bimolecular reaction

$$E + I \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} EI$$

and the values for the rate constants, k_{12} and k_{21}, were determined from eq 2 to be $k_{12} = 1.5 \times 10^6$ M^{-1} sec^{-1} and

$k_{21} = 4$ sec^{-1}. The value for the kinetically defined dissociation constant, $K_s = k_{21}/k_{12} = 2.6$ μM is in agreement with the value from the titration experiments. The kinetic constants were found to be indeed similar to those observed for L-isoleucine, namely, $k_{12} = 2.2 \times 10^6$ M^{-1} sec^{-1} and $k_{21} = 15$ sec^{-1} (Holler and Calvin, 1972).

pH Dependence for L-Isoleucine Binding and Fluorescence Quenching. The following investigation was designed to collect information about ionizing groups which are somehow involved in substrate binding and fluorescence quenching. The dissociation constant for the binding of L-isoleucine and the fluorescence intensities of the enzyme–TNS complex and of the enzyme–TNS–L-isoleucine complex were measured over the pH range 7–10 at a constant ionic strength of 0.05 M (Figure 6). We believe that no pH-dependent denaturation occurs in this range for the following reasons. (1) Fluorescence of the protein when excited at 280 nm has been reported to be sensitive to denaturation (Penzer et al., 1971). Thus, protein fluorescence may be used to determine when denaturation occurs. Upon increasing pH, we found that fluorescence became unstable only when pH 10 was exceeded. (2) The degree of fluorescence at saturating concentrations of L-isoleucine was found to be invariant against pH. This observation would hardly be consistent with a pH-induced denaturation.

It is seen from Figure 6 that fluorescence of the enzyme–TNS complex begins to decrease when pH 8.5 is exceeded. Similarly, the stability of the enzyme–L-isoleucine complex, as reflected by the value of K_{Ile}, remains constant up to pH 8.5, and then decreases. The fluorescence of the enzyme–TNS–L-isoleucine is invariant against pH and apparently coincides with fluorescence of the enzyme–TNS complex at pH 10. The observations are consistent with a pK(app) = 9.3–9.5 for an ionization of a group involved in the L-isoleucine-induced fluorescence quenching. The pH profile for the dissociation constant is too incomplete to decide whether it is determined by the same pK(app).
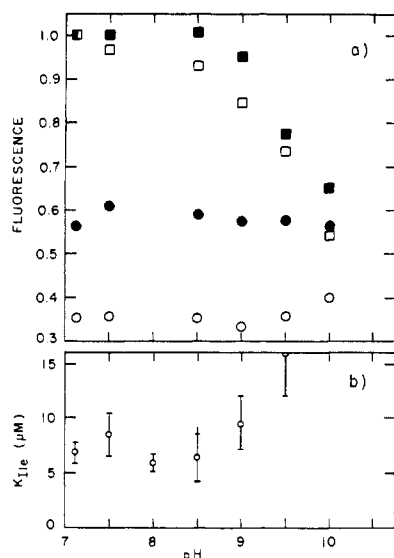
FIGURE 6: Fluorescence intensity of Ile-tRNA synthetase–TNS and of the Ile-tRNA synthetase–TNS-L-isoleucine complex (a) and the stability of the Ile-tRNA synthetase–TNS-L-isoleucine complex (b) as a function of the pH of the reaction mixture at 25°. Squares refer to the enzyme–TNS complex and circles to the enzyme–TNS-L-isoleucine complex. Excitation at 290 nm is indicated by open symbols and excitation at 366 nm by filled symbols. Emission was observed at 470 nm. The values for the fluorescence intensity are normalized to those at pH 7.25. Initial concentrations were 0.18 μM enzyme, 9 μM TNS, 4 μM to 2.4 mM L-isoleucine, 0.05 M Tris-HCl, and sufficient NaCl to provide a constant ionic strength of 0.05 M. Note the stronger quenching for excitation at 290 nm, giving evidence for a substrate-induced conformation change (Holler *et al.*, 1971).

TABLE II: Interaction of ATP and Related Compounds with Ile-tRNA Synthetase at pH 8.0.[a]

Compound	Method	K_i (mM)	K_m (mM)	K_s (mM)	Max Quenching[b]
ATP	F			0.25 ± 0.03	1.0
	P[c]		0.15		
	F (37°)			0.52^d	
	P (37°)		0.42^d		
AMP	F			0.75 ± 1	0.6
	P (37°)	0.9^d			
Adenosine	P (37°)	0.25^d			
Adenine	P (37°)	30^d			
D-Ribose	P (37°)	$17^{d,e}$			
Pyrophosphate	F			0.26 ± 0.07	1.0
	P[c]		0.03		

[a] Investigation at 25° unless otherwise stated. The same comments apply as for Table I. [b] Standard deviations are 10–15%. [c] Cole and Schimmel, 1970. [d] Experimental error was not calculated. [e] Based on assumptions that only the β-furanose form is inhibitory and that this form comprises 18% of the D-ribose in an aqueous solution at equilibrium (Angyal and Pickles, 1967).

ATP and Related Compounds. The interaction of ATP and related compounds with Ile-tRNA synthetase was investigated in a fashion similar to that for L-isoleucine and related compounds. The results from ATP-[^{32}P]PP$_i$ exchange and titration experiments are summarized in Table II. Inhibition constants were measured against ATP. AMP, adenosine, adenine, and D-ribose were found to be competitive inhibitors. A typical Lineweaver–Burk plot (Lineweaver and Burk, 1934) is shown for ATP, adenosine, and adenine in Figure 7. Some of the observations for the L-isoleucine related compounds were repeated here. (1) The values for the Michaelis–Menten constants are similar to the values of the dissociation constants as determined from fluorescence titration. (2) The degree of fluorescence quenching is the same for L-isoleucine, ATP, and pyrophosphate.

When the inhibition constant of adenosine is compared with those of its components, adenine and D-ribose, we find that the K_i(app) for adenosine is approximately equal to the product of the K_i(app) values for the latter two compounds, indicating the likelihood that the adenyl and ribosyl moieties of adenosine bind essentially independently of one another.

When we compare the binding of ATP with its components, AMP and pyrophosphate, however, we discover that all three bind with roughly equivalent affinities. Thus the dissociation constant of ATP is much greater than would have been predicted from the dissociation constants of the subunits.

Discussion

Ile-tRNA synthetase very likely exists as a monomer in aqueous solutions under conditions similar to those used in

our experiments (Baldwin and Berg, 1966; Arndt and Berg, 1970; Berthelot and Yaniv, 1970). One molecule of L-isoleucine (Berthelot and Yaniv, 1970) or one molecule of L-isoleucyl adenylate (Baldwin and Berg, 1966) was found to combine with one molecule of enzyme, suggesting one active site per protein molecule. This conclusion is in accord with our finding that kinetic and equilibrium investigations are interpretable on basis of a single site for each ligand under study.

Structural Components of the L-*Isoleucine Specific Site.* Loftfield and Eigner (1966) have discussed the involvement of hydrophobic forces in the specific enzyme-L-isoleucine interaction. Further evidence has been reported by Holler and Calvin (1972) on the basis of thermodynamic properties. Particular information is gained by comparison of the values for the dissociation and Michaelis–Menten constants of compounds with varying structures of the side chain. Removal of the 3-methyl group of L-isoleucine as in α-DL-aminopentanoic acid is associated with a 400-fold increase of the value for the dissociation constant, equivalent with the loss of approximately 3.6 kcal for the standard free energy of complex formation. (No correction is made for the D enantiomer. If the D enantiomer does not bind, the actual loss is smaller by 0.4 kcal.)

Similarly, when the methyl group of the longer tail of the L-isoleucine side chain is removed, generating L-valine, 2.2 kcal is lost. We conclude that both the methyl and the ethyl groups of L-isoleucine bind to subsites which are both hydrophobic. Consequently, when the methylene group of the ethylene subsite is replaced by oxygen, the extent of hydrophobic interaction is decreased as indicated by the loss of 1.9 kcal of standard free energy as evaluated for O-methyl-threonine. The increments for hydrophobic binding exceed by far what is known from analysis of enzyme–ligand interactions. Heidberg *et al.* (1967) have investigated in detail the hydrophobic interaction between 1-*n*-alkylamines and the active site of trypsin. They have determined the incre-

ments to be 1.5 kcal for the methyl group and 1.1 kcal for the methylene group, the same values as have been found for the transfer of hydrocarbons from water to nonpolar solvents (Kauzmann, 1959; Nemethy and Scheraga, 1962). A similar conclusion has been put forward by Loftfield and Eigner (1966).

It was of further interest to determine whether the two hydrophobic subsites, which may be considered as pockets or slots, were closed at their ends, as is the case for trypsin and chymotrypsin (Steitz et al., 1969). When the n-alkyl side chain of the α-aminopentanoic acid is elongated we expect one of two alternative responses. Either the elongated tail extends beyond the end of the slot if it is open, or the α-amino group and the carboxylic group are pushed out of their places into catalytically improper positions if the end of the slot is closed. For the first alternative, we would expect that the elongated substrate maintains the same level of ATP–[^{32}P]PP$_i$ exchange as observed for α-aminopentanoic acid and that the Michaelis–Menten constant would be almost invariant. For the second alternative, a substantial decrease in the rate of exchange and binding affinity would be expected. Comparison of the data for α-DL-aminopentanoic acid, α-DL-aminohexanoic acid, and α-DL-aminoheptanoic acid indicates that both parameters were only slightly changed, suggesting that one, if not both, ends of the subsites are not rigidly closed.

The results of the removal of the α-amino or carboxylic group are intriguing. When they were both removed and the carboxylic group was replaced by an alcohol or aldehyde group the affinity for forming complexes with Ile-tRNA synthetase dropped by 3.7 kcal for the 3-methylpentyl-1-derivatives and by 4.8 kcal for 2-methyl-1-butanol. The different values indicate a weak interaction for the alcohol or aldehyde group of the 3-methylpentyl-1 derivatives. However, when this group becomes a carboxylic group, interaction is abolished. It is possible that the alcohol or (hydrated) aldehyde group interacts via a hydrogen bond with the enzyme and that the abolishing effect of a carboxylic group is an electrostatic repulsion directed from a negatively charged group at the active site. Possibly this negative charge could interact favorably with the α-ammonium group of the amino acids, contributing the 3.7 kcal missing for the neutral 3-methylpentyl-1 derivatives. However, we learn from the dissociation constants for L-isoleucinol and 2-methyl-1-butylamine (in the absence of co-ligands) that our model is incomplete or incorrect. Comparing L-isoleucine with L-isoleucinol and 2-methyl-1-butylamine (in the absence of co-ligands) we find that stability of the enzyme–ligand complexes is decreased by 4.1 and 4.5 kcal, respectively. Thus, incorporation of the α-amino group alone does not enhance the binding affinity. Yet, in the specific substrate L-isoleucine the α-amino group must contribute at least 4.5 kcal, the only difference being that the carboxylic group is present. Apparently, both groups must be involved simultaneously in order to provide a maximum of interaction. A sensible approach to mimic the electrostatic effect of the carboxylic group seemed to us to provide an additional external negative charge, for instance, by binding ATP, AMP, or pyrophosphate to their specific sites. The outcome of the experiments is in accord with our assumption. When ATP is present as a co-ligand, 2-methyl-1-butylamine binds as strongly as L-isoleucine. The effects for AMP and pyrophosphate are gradually weaker. Similarly, L-isoleucinol binds 60 times more strongly under conditions of ATP–[^{32}P]PP$_i$ exchange than in the absence of co-ligands. The similarity of the interactions of 2-methyl-1-butylamine and L-isoleucine

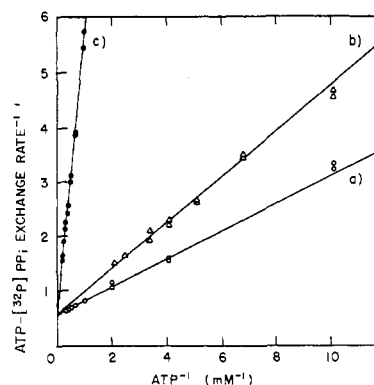FIGURE 7: Lineweaver–Burk plot for the rate of ATP–[^{32}P]PP$_i$ exchange as a function of the ATP concentration in the presence of inhibitors, 37°, pH 8.0. Initial concentrations were 1.2 nM Ile-tRNA synthetase, 2 mM L-isoleucine, 2 mM sodium pyrophosphate, 10 mM KF, 0.1 M Tris-HCl, 0.01 M 2-mercaptoethanol, 0.05–5 mM ATP. The reaction mixture contained (a) no inhibitor, (b) 10 mM adenine, and (c) 5.5 mM adenosine. The concentration of MgCl$_2$ was varied concomitantly with the concentration of ATP to provide a 1 mM excess of magnesium over the total concentration of ATP plus pyrophosphate.

with Ile-tRNA synthetase includes also the kinetic constants, as is indicated in Figure 5, and presumably also the type of mechanisms, which was found to be a two-step process for L-isoleucine (Holler and Calvin, 1972).

The observation that L-isoleucine-related compounds, lacking either the α-ammonium group or the carboxylic group, are poor inhibitors, if at all, together with the finding that ATP, AMP, and pyrophosphate promote the interaction between the active site and an inhibitor lacking the carboxylic group, can be understood on the basis of the following tentative model. One of the structural components of the active site is an ion pair. Binding of L-isoleucine is associated with an opening of the pair and formation of new pairs at the α-ammonium group and at the carboxylic group, respectively, of the substrate. The positive charge paired with the substrate carboxylic group may simultaneously interact with the oligophosphate group of ATP. Since we have found that binding of L-isoleucine and ATP is independent (Holler et al., 1971), this positively charged group must have a similar position in both enzyme–ligand complexes. The opening of the ion pair could be the initiation of the conformation change which was observed to follow the attachment of L-isoleucine to the enzyme (Yarus and Berg, 1969; Holler et al., 1971; Holler and Calvin, 1972). The ion pair is also opened when, for instance, ATP binds to its specific site. The rate of the opening (and the conformation change) varies with the extent of the side chains of both the L-isoleucine-related compounds and the ATP-related compounds. In the case when the carboxylic group is lacking, as for L-isoleucinol and 2-methyl-1-butylamine, the open ion pair is poorly stabilized, presumably because positively charged groups approach each other too closely. However, when the open conformation has already been stabilized by ATP or related compounds, attachment of the inhibitor is easy.

The extent of the conformation change is reflected in the degree of fluorescence quenching induced by binding of the various ligands. From Tables I and II we have evidence that the extent is invariant against the structure of the compound in the binary enzyme–ligand complex. Thus, neutral L-iso-
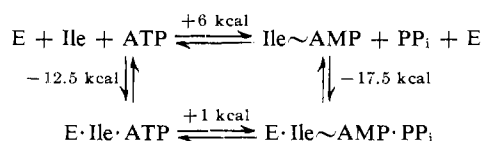
leucine-related compounds induce the same degree of quenching as do L-isoleucine, ATP, AMP, and pyrophosphate. Apparently, the hydrophobic binding is rigidly coupled to the opening of the ion pair. Moreover, the rigidity of the substrate specific site is indicated by only a threefold decrease in the maximum rate of ATP-[^{32}P]PP$_i$ exchange as compared with a 3000-fold difference between the values for K_m(app) when L-isoleucine and α-DL-aminoheptanoic acid are compared (Table I). Furthermore, rigidity may be the reason why methylation or guanidation of the α-amino group is associated with a complete loss of binding ability. Apparently, binding of the modified ammonium group is prohibited by steric hindrance, thus concomitantly preventing binding at the rigidly coupled hydrophobic site. As has been pointed out by Loftfield and Eigner (1966), this rigidity is in severe contrast to the sensitivity of the rate of hydrolysis catalyzed by chymotrypsin and trypsin as a function of the stability of the enzyme–substrate complexes.

From the description of our model an easy test of the ion-pair hypothesis is suggested. It must be possible to open the ion pair simply by pH titration, by neutralizing the positive component. We have done the experiment, Figure 6, following the fluorescence properties of the enzyme–TNS and the enzyme–TNS–L-isoleucine complex. In accord with our hypothesis, we found that fluorescence quenching is produced on increasing pH, ultimately reaching that produced by the binding of a substrate. The apparent pK of 9.5 is in agreement with the ionization of an amino group.

In summary, we have presented evidence for the existence of a hydrophobic binding site for L-isoleucine consisting of two subsites to take up the methyl group and the ethyl group, respectively, of the aliphatic side chain. One or both subsites may be open at the ends. The strength of binding to these subsites cannot be accounted for on the basis of hydrophobic interactions alone but presumably reflects a coupling to secondary interactions. It is supposed that the hydrophobic binding is rigidly linked to the opening of an ion pair. The opening is easily accomplished with L-isoleucine, ATP, or AMP, but appears to be difficult with L-isoleucinol, 2-methyl-1-butylamine, or 3-methylpentanoic acid, presumably because of electrostatic repulsion. The strong inhibition observed for L-isoleucinol and 2-methyl-1-butylamine as observed in the ATP-[^{32}P]PP$_i$ exchange reaction is coupled to the binding of ATP, whereas the binding of L-isoleucine apparently is not. We think that care has to be taken when the binding of an amino alcohol analog of an amino acid is used as a tool to investigate the kinetic pathway of the catalytic reaction as has been done in the case of the leucyl activating enzyme from *E. coli* (Rouget and Chapeville, 1971).

Free-Energy Considerations for the Ile-tRNA Synthetase–ATP Complex. We have recently presented evidence (Holler and Calvin, 1972) that formation of L-isoleucyl adenylate from L-isoleucine and ATP is promoted by approximately 5 kcal when substrates and products are bound to Ile-tRNA synthetase as compared to the reaction in the free solute state. The relation between the standard free energy for the reaction of L-isoleucine and ATP and the standard free energy of the reaction when they are bound to the enzyme is described by the following equation

$$E + \text{Ile} + \text{ATP} \underset{-12.5 \text{ kcal}}{\overset{+6 \text{ kcal}}{\rightleftharpoons}} \text{Ile}\sim\text{AMP} + \text{PP}_i + E$$

$$\text{E} \cdot \text{Ile} \cdot \text{ATP} \underset{}{\overset{+1 \text{ kcal}}{\rightleftharpoons}} \text{E} \cdot \text{Ile}\sim\text{AMP} \cdot \text{PP}_i \qquad -17.5 \text{ kcal}$$

It is seen that the L-isoleucyl adenylate formation is promoted by complex formation with the reactants because the standard free energy of binding both substrates is overcompensated by the standard free energy of binding both products. One possible explanation is that ATP takes up free energy upon binding to the enzyme resulting in partial distortion or strain of those parts of the substrate which have to undergo reaction. As indicated in Table II, each of the structural components of ATP (*i.e.*, AMP and pyrophosphate) binds separately to the enzyme with an affinity comparable to that of ATP itself. Linked together as ATP, these components tend to come as close as possible into contact with their particular binding sites in order to achieve a maximum of interaction. This tendency apparently forces the ATP into a strained configuration. The final result is a compromise between the intensity of ligand–enzyme interaction and the degree of distortion or "strain" of the ATP molecule. As an estimate of the free energy taken up in the distortion, we may consider the difference between the free energy of binding of ATP and the sum of the binding energies of AMP and pyrophosphate as determined from the dissociation constants. Thus, $\Delta G_{\text{dist}} = \Delta G_{\text{ATP}} - (\Delta G_{\text{AMP}} + \Delta G_{\text{PP}_i}) = -5 \text{ kcal} + 4.3 \text{ kcal} + 5 \text{ kcal} = 4.3 \text{ kcal}$. Of course, this calculation can only be regarded as a crude approximation. Nevertheless, the value of 4.3 kcal for ΔG_{dist} is in agreement with the estimated 5 kcal by which the enzyme-mediated L-isoleucine activation is favored over the non enzymic reaction.

We found that adenosine is bound somewhat more strongly than AMP. A tight binding of moieties on both sides of the residue undergoing reaction is exactly what one would anticipate for the effectiveness of a distortion in enhancing reactivity. Since it is the α-phosphate of ATP which undergoes nucleophilic substitution in the reaction, we would have indeed expected the adenosine and the β,γ-pyrophosphate to be bound quite strongly to the enzyme.

We have tentatively proposed that ATP assumes a high energy state when bound to the enzyme. A high energy state of the substrate has been proposed for the hydrolysis of chitohexose by lysozyme on the basis of crystallographic and chemical evidence (Blake *et al.*, 1967; Rupley and Gates, 1967; Rupley *et al.*, 1967). The hypothesis may be of general interest. Santi *et al.* (1971) have reported values of the Michaelis–Menten and inhibition constants for the interaction of ATP, AMP, adenosine, and pyrophosphate with Phe-tRNA synthetase from *E. coli* which are consistent with the hypothesis.

The ATP-[^{32}P]PP$_i$ exchange reaction of the L-isoleucine system requires magnesium ions. We believe that the function of magnesium may be understood mainly in terms of orientation of the pyrophosphate moiety within the Michaelis–Menten complexes and possibly as a stabilizer of the transition state, as has been discussed by Santi *et al.* (1971). Experiments are underway to elucidate this requirement and to add support to our hypothesis.

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