Submitted to
Biochemistry

LBL-939
Preprint

ISOLEUCYL-tRNA SYNTHETASE OF E. coli B. A RAPID KINETIC INVESTIGATION OF THE L-ISOLEUCINE ACTIVATING REACTION

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May 1972

AEC Contract No. W-7405-eng-48

Isoleucyl-tRNA Synthetase of E. coli B. A Rapid Kinetic Investigation of the L-Isoleucine Activating Reaction*

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Running title: Preequilibrium Kinetics of L-Isoleucyl-tRNA Synthetase

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Supported by U. S. Atomic Energy Commission and a Helen Hay Whitney fellowship (E.H.).

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ABSTRACT: We have investigated the preequilibrium kinetics of the L-isoleucine activation reaction catalyzed by Ile-tRNA synthetase in the presence of a fluorescent reporter group, 2-p-toluidinylnaphthalene-6-sulfonate, using the stopped-flow technique. It is found that of all the reactants involved, L-isoleucine binds slowest to the enzyme, apparently in a two-step process. The kinetics of the reaction are invariant in the presence of co-reactants, whereas the kinetics for ATP are drastically changed in the presence of Mg^{2+} ions.

The formation of enzyme bound L-isoleucyl∿AMP is conveniently followed at dilute concentrations. The value for the rate constant of formation was determined to be 135 sec^{-1} and of the reverse process to be 670 sec^{-1} at pH 8.0 25°C. These values are considerably higher than the rate constant 15 sec^{-1} of the dissociation reaction for L-isoleucine. The value of the kinetically defined equilibrium constant between the ternary Michaelis-Menten complex and the ternary enzyme-product complex indicates that, at equilibrium, the Michaelis-Menten complex is favored.

The effect of temperature has been determined, and a tentative interpretation of the thermodynamic parameters is offered. The zero standard enthalpy and positive entropy for binding of L-isoleucine is consistent with hydrophobic interactions, whereas the enzyme-ligand complexes for ATP and pyrophosphate might be stabilized by hydrogen-bonds and ion-ion interactions. The equilibrium constant of formation of the ternary enzyme-product complex from the Michaelis-Menten complex does not increase significantly with temperature.

The types of kinetic pathways have been restricted to the alternative of a random mechanism or an ordered sequential mechanism in which L-isoleucine binds first. We believe that the mechanism is random.

It is almost habit to begin a paper about the mechanism of action of aminoacyl-tRNA synthetases with the two following oversimplified equations characterizing their mode of action.

$$E^1 + AA + ATP \underset{}{\overset{Mg^{2+}}{\rightleftharpoons}} E \cdot AA \sim AMP + PP_i \qquad (1)$$

$$E \cdot AA \sim AMP + tRNA^{AA} \rightleftharpoons E + AMP + AA - tRNA^{AA} \qquad (2)$$

Equation (1) describes the amino acid activation and equation (2) the aminoacyl transfer to the cognate tRNA. In this paper we shall be concerned with only the first reaction. The L-isoleucine activation system from E. coli has been thoroughly investigated by Cole and Schimmel (1970) with use of the ATP-[^{32}P]PP_i exchange assay and in part by Penzer, Bennett and Calvin (1971) by use of substrate-sensitive denaturation studies. Although it should be possible to measure kinetics for aminoacyl-tRNA synthetases which have substrate induced fluorescence properties (Hélène, Brun and Yaniv, 1971; Blanquet, Fayat, Waller and Iwatsubo, 1972), using equilibrium perturbation techniques (Eigen and DeMaeyer, 1963), and transient kinetic methods (Gutfreund, 1971), preequilibrium kinetics have been scarcely investigated (Blanquet et al., 1972). Ile-tRNA synthetase (E. coli B) has fluorescence properties which are inert against substrate binding (Penzer et al., 1971). Yet, we have recently shown that we can provide equivalent information by supplying the system with TNS, a reversibly binding, fluorescent reporter group (Holler, Bennett and Calvin, 1971). In this paper, we have investigated the preequilibrium kinetics of the L-isoleucine activation step as catalyzed by Ile-tRNA synthetase with use of stopped-flow techniques.

[1]Abbreviations used are: E, an AA-tRNA synthetase, in particular Ile-tRNA synthetase; E·AA~AMP, enzyme-aminoacyl adenylate complex; TNS, 2-p-toluidinylnaphthalene-6-sulfonate; E:Ile, enzyme-L-isoleucine complex; E·ATP, enzyme-ATP complex.

Materials and Methods

Ile-tRNA synthetase was obtained as 300- to 350-fold purified preparations from E. coli B cells (Miles Laboratories) by the method of 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 to 750 units/mg, where one unit is defined as the formation of 1 μmole of $[^{32}P]ATP$ from $[^{32}P]PP_i$ in 15 min at 37°C in the standard assay mixture (Baldwin and Berg, 1966). The published value of the molecular weight (112,000, Baldwin and Berg, 1966) was used to calculate the enzyme concentration. $[^{32}P]PP_i$ was prepared from $[^{32}P]P$ (International Chemical and Nuclear Corp.) as described by Berg (1958). L-Isoleucine was obtained from Cyclo, Na_2ATP from Nutritional Biochemical Corp., and TNS from Sigma (Lot 60C-5270). 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.

Fluorescence measurements. The measurement of dissociation constants was performed by the method already described (Holler, Bennett and Calvin, 1971). In principle, the degree of fluorescence quenching for enzyme-bound TNS is measured as a function of ligand concentration.

Excitation light was either of 290 nm (tryptophan) or 366 nm (TNS) wavelength. Emission was observed at 470 nm. Slits for excitation and emission were 8 nm and 10 nm, respectively. Quartz cells of 4 mm x 10 mm dimensions were used with the excitation light entering through the 4 mm window. Fluorescence was measured on a Hitachi Perkin-Elmer Model MPF-2A fluorescence spectrophotometer. The sample compartment was thermostated at a constant temperature within $\pm$ 0.5°C and was flushed with nitrogen.

The reaction solutions were 0.05 M Tris-HCl or 0.02 M sodium phosphate of pH 8.0 and contained 0.01 M 2-mercapto ethanol as SH-protecting reagent. Concentrations of TNS varied between 5 µM and 20 µM and of Ile-tRNA synthetase between 0.05 µM and 0.2 µM. Saturation curves were obtained by adding aliquots of 0.5 µl or 1 µl of the buffer solution containing the ligand under study. The titration curves were corrected for dilutions. No correction for the inner filter effect of the ligands was made; in case of ATP only the excitation light at 366 nm was used.

Kinetic measurements. Kinetics were followed by observing the time dependent fluorescence change after rapid mixing of the reactants in a modified Durrum-Gibson stopped-flow spectrofluorimeter. The apparatus was equipped with a Tektronix 564 storage oscilloscope, a 2A63 differential amplifier and a 3B3 time base. Excitation light was provided by a 75 watt xenon lamp. After passing the Durrum monochromator, light of 290 nm entered the 20 mm reaction cell as it is set up for absorbance measurements. Light intensity was observed at 90° to the incident beam through a cut-off filter (Corning #373) and measured

by a photomultiplier (Du Mont 6365). The preamplified signal entered the Durrum temperature jump electronic unit (D-150). The dead time of the apparatus was 4 msec. For each tracing, 0.3 ml of a buffered solution of enzyme (0.10 μM to 0.2 μM) was mixed with an equal volume of the same buffer containing the ligands under study, unless otherwise stated. Both solutions contained TNS at equal concentrations. The reaction dependent fluorescence change was displayed on the screen of the storage oscilloscope. The rate constants k_{obs} were evaluated from the photographs by measuring the slopes of the first order plots. Reactions were examined over the temperature range from 9°C to 37°C. Tris-HCl (0.05 M) or sodium phosphate (0.02 M) of pH 8.0 were used as buffer. Rate constants were independent of the kind of buffer applied. In experiments where L-isoleucine or ATP had to be preincubated with enzyme, EDTA, 1 mM, was added to the solution. Catalytic conditions may be obtained by applying $MgCl_2$ in excess of EDTA to the second solution of reactants to be mixed with the preincubated mixture.

For some of the ligands, the overall change of fluorescence intensity associated with the observed reaction was compared with the change at equilibrium condition. To obtain the overall change from the reaction trace, the first order plot was extrapolated back beyond zero time for the dead time of the apparatus. The total change of the fluorescence intensity between this time and equilibrium is taken as the overall change of fluorescence intensity. To obtain the difference of the intensity at equilibrium (before and after mixing), the total intensity of the enzyme-ligand mixture was measured at equilibrium and compared with the sum of the intensities for separate 1:1 mixtures of enzyme and ligand with buffer.

Effect of temperature. Thermodynamic standard parameters were evaluated from the temperature dependence of dissociation constants and kinetic constants. The standard enthalpy ΔH^o of formation of a complex between Ile-tRNA synthetase and a ligand was determined from the slope of the Van't Hoff-plot. The standard free enthalpy was calculated from the dissociation constant (25°C) according to $\Delta G^o = 2.3\ RT \log K_{ligand}$ and the standard entropy, ΔS^o, according to $\Delta S^o = (\Delta H^o - \Delta G^o)/T$.

Thermodynamic standard parameters of activation were calculated from the activation energy, E_a, and the frequency factor, A, of the Arrhenius plots (Glasstone, Laidler and Eyring, 1941). The following equations were used:

$$\Delta H^{o\ddagger} = E_a - RT$$

$$\Delta S^{o\ddagger} = R(2.3 \log A - 2.3 \log \frac{RT}{Nh} - 1)$$

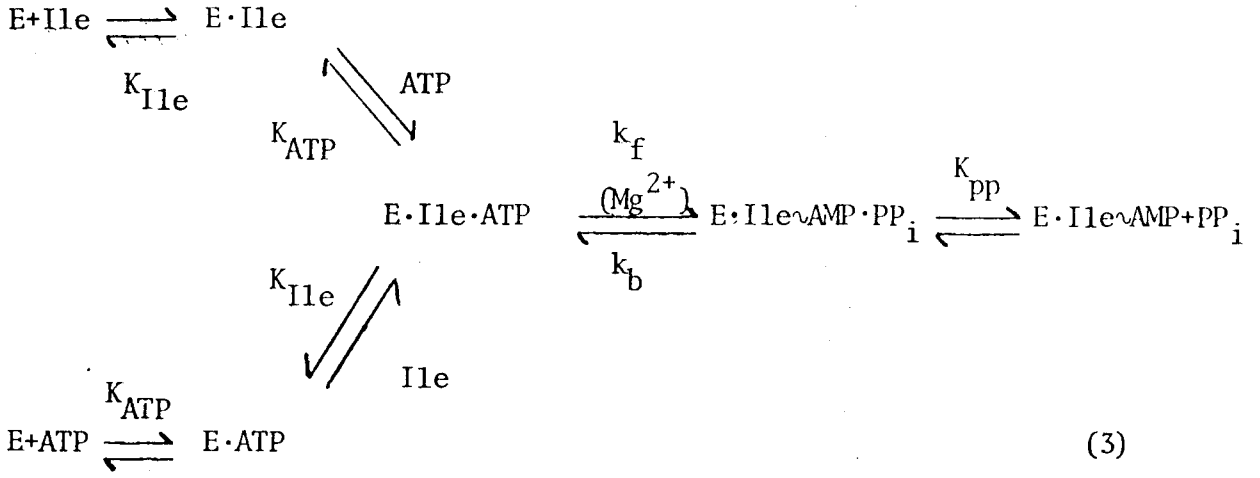
The symbols R, T, N, h refer to the gas constant, absolute temperature, Avogadro's number and Planck's constant, respectively. At 25°C, the equation is written as $\Delta S^{o\ddagger} = 4.6 \log A - 61$ (e.u.).

Treatment of the kinetic data. Recently, TNS has been used as a fluorescent reporter group for Ile-tRNA in measuring binding of the various ligands involved in the amino acid activation reaction (Holler, Bennett, and Calvin, 1971). We have found that TNS does not interfere with the action of the enzyme in the ATP-$[^{32}P]PP_i$ exchange reaction (Holler, Bennett, and Calvin, 1971) nor with the tRNA charging reaction (Holler et al., manuscript in preparation). Moreover, we have examined the kinetics following rapid mixing of solutions of enzyme (0.14 μM) and TNS (5 μM to 90 μM) in the stopped-flow apparatus. We were not able to detect any time dependent process which could have been attributed to

enzyme-TNS interaction. Thus, interaction properties between the enzyme and the various ligands are evaluated from kinetic and equilibrium data without including consideration of enzyme-TNS interaction. In principle, a reaction between enzyme and a ligand will be associated with a change of fluorescence of the enzyme bound TNS as if this would be an intrinsic property of the enzyme. An observed change, ΔF, of fluorescence intensity will be proportional to the concentration of an enzyme-ligand complex. Similar considerations can be applied as in dealing with changes of absorbance.

Before deriving rate equations, the steady-state kinetic mechanism for the amino acid activation reaction catalyzed by Ile-tRNA synthetase will be considered. Cleland (1970) has characterized two alternative types for the bireactant mechanism, the random and the ordered type. The sequential mechanism of the ordered type requires that a particular substrate combines first with the enzyme. If the second substrate binds first, a ternary enzyme-substrate complex cannot be formed or, if it is formed, it will be unproductive.

At the moment, there is no clear evidence for the kind of mechanism for Ile-tRNA synthetase. Cole and Schimmel (1970) have attempted to correlate the rate of the ATP-[^{32}P]PP_i exchange reaction with the pathways for random or ordered mechanisms. However, they came to the conclusion that there was no clear-cut answer. Holler, Bennett, and Calvin (1971) have evidence that the dissociation constants for reactants (of the activation reaction) are independent of binding of co-reactants, thus indicating that the random mechanism may be probable. In conclusion, we may formulate the reaction scheme of the amino acid activation in the following way:



(3)

Substrates and enzyme can form the ternary complex E·Ile·ATP either in an ordered pathway, by following only one or the other branch, or in a random sequence, following both branches. The ternary enzyme-substrate complex turns slowly over to form a ternary enzyme-product complex E·Ile∿AMP·PP_i. The association-dissociation equilibria involving L-isoleucine, ATP and pyrophosphate are supposed to be in preequilibrium with respect to the rate controlling amino acid activation step. As it will turn out, this assumption is only valid at dilute concentrations of reactants [$(Ile)_o < K_{Ile}$, $(ATP)_o < K_{ATP}$, $(PP_i)_o < K_{pp}$]. Dissociation of L-isoleucyl∿AMP is extremely slow (Norris and Berg, 1964; Eldred and Schimmel, 1972) and needs not be considered for the length of time under observation. Derivation of expressions for the observed rate constant will not distinguish whether the kinetic pathway is ordered or random under the conditions, except for the case when enzyme and both substrates could combine in the wrong order of sequence to form an unproductive ternary enzyme-substrate complex. At saturation condition for both substrates, the reaction rate would be only half of that observed for dilute solutions.

As a first approach, we shall calculate the expression for k_{obs} in considering all association-dissociation processes as being in rapid preequilibrium. When we mix a solution of enzyme with a solution containing the reactants, we observe a change of fluorescence intensity which will be associated with the formation of enzymically bound L-isoleucyl∿AMP. The reaction rate is described by equation (4):

$$\frac{d\Delta F}{dt} \triangleq \frac{d}{dt}\left[(E\cdot Ile\sim AMP)+(E\cdot Ile\sim AMP\cdot PP_i)\right] = k_f(E\cdot Ile\sim ATP) - k_b(E\cdot Ile\sim AMP\cdot PP_i) \tag{4}$$

We elaborate equation (4) by including the following equations and conditions:

The concentration of reactants does not decrease during reaction,

$$(Ile)_o,\ (ATP)_o,\ (PP_i)_o \gg (E)_o \tag{5}$$

(subscript zero defines initial concentrations)

$$(E)_o = (E) + (E\cdot Ile) + (E\cdot ATP) + (E\cdot Ile\cdot ATP) + (E\cdot Ile\sim AMP\cdot PP_i) + (E\cdot Ile\sim AMP) \tag{6}$$

$$K_{Ile} = \frac{(E)\cdot(Ile)_o}{(E\cdot Ile)} \qquad K_{Ile}\,K_{ATP} = \frac{(E)\cdot(Ile)_o\cdot(ATP)_o}{(E\cdot Ile\cdot ATP)}$$

$$K_{ATP} = \frac{(E)\cdot(ATP)_o}{(E\cdot ATP)} \qquad K_{PP} = \frac{(E\cdot Ile\sim AMP)\cdot(PP_i)_o}{(E\cdot Ile\sim AMP\cdot PP_i)} \tag{7}$$

Introducing equations (6) and (7) into equation (4) and using condition (5) we obtain

$$\frac{d}{dt}(E\cdot Ile\sim AMP) = k_f\cdot(E)_o\cdot G\,\frac{K_{PP}}{K_{PP}+(PP_i)_o} - (E\cdot Ile\sim AMP)\left[k_f\cdot G + k_b\,\frac{(PP_i)_o}{K_{PP}+(PP_i)_o}\right] \tag{8}$$

with $$G = \left\{\frac{[(Ile)_o+K_{Ile}][(ATP)_o+K_{ATP}]}{(Ile)_o\cdot(ATP)_o} + \frac{K_{Ile}\cdot K_{ATP}}{K_{PP}}\,\frac{(PP_i)_o}{(Ile)_o\cdot(ATP)_o}\right\}^{-1}$$

From equation (8) the expression for k_{obs} is derived in a straightforward manner:

$$k_{obs} = k_f \cdot G + k_b \frac{(PP_i)_o}{K_{PP} + (PP_i)_o} \qquad (9)$$

We found that equation (9) worked well for conditions of dilute solutions of substrates [$(Ile)_o < K_{Ile}$, $(ATP)_o < K_{ATP}$]. At higher concentration, however, the kinetics interfered with the slow formation of complex E·Ile. The problem could be bypassed by preincubating enzyme plus L-isoleucine. However, in cases when we wished to study the dependence of the kinetics on L-isoleucine concentrations [$(Ile)_o \gtrsim K_{Ile}$] we had to apply a second approach. In particular, we chose to preincubate enzyme with ATP at saturating concentrations [$(ATP)_o >> K_{ATP}$]. Conditions were furthermore simplified by using dilute concentrations of enzyme [$(E)_o << K_{PP}$] and omitting pyrophosphate from the reaction mixture. As we shall demonstrate, the reverse reaction of formation of the intermediate does not contribute significantly to the observed reaction rate under these conditions. Instead of equation (3) we consider reaction scheme (10):

$$E\cdot ATP + Ile \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} E\cdot ATP\cdot Ile \xrightarrow[(Mg^{2+})]{k_f} E\cdot Ile\sim AMP + PP_i \qquad (10)$$

Applying the steady-state approximation to the concentration of the ternary enzyme-substrate complex, E·ATP·Ile, we calculate the following expression for the observed rate constant:

$$k_{obs} = k_f \frac{(Ile)_o}{\frac{k_{21} + k_f}{k_{12}} + (Ile)_o} \qquad (11)$$

The assumptions made here to derive equations (9) and (11) are of fundamental importance, and they will receive attention as part of the results of our investigation.

Results

Binding of ligands at non-catalytic conditions. Binding kinetics for each of the ligands L-isoleucine, ATP, pyrophosphate and Mg^{2+} were at first investigated separately and finally in the presence of other ligands--however, without establishing the catalytic conditions, that is, not having L-isoleucine, ATP and Mg^{2+} present in the same solution.

L-Isoleucine. When up to 0.32 mM L-isoleucine was mixed with 0.15 μM of enzyme in the stopped-flow apparatus, a process was observed which was indicated as quenching of fluorescence. A typical oscilloscope display is shown in Figure 1. When, at first at 25°C, the rate constants k_{obs} of the reaction were investigated as function of the concentration of L-isoleucine, a linear plot was obtained, Figure 2a. The type of plot would indicate that complex formation between enzyme and L-isoleucine proceeds via a bimolecular process (Czerlinski, 1966) and is described by equation (12).

$$E + Ile \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} E \cdot Ile \qquad (12)$$

The expression for k_{obs} is (Czerlinski, 1966)

$$k_{obs} = k_{21} + k_{12} (Ile)_o \qquad (13)$$

The rate constants were evaluated from slope and intercept and are $k_{12} = 2.2 \cdot 10^6\ M^{-1}\ sec^{-1}$ and $k_{21} = 15\ sec^{-1}$. The kinetically defined dissociation constant $K_{Ile} = k_{21}/k_{12}$ was calculated to be 6.8 μM, in

agreement with the value 5.8 μM evaluated from titration experiments (Holler, Bennett and Calvin, 1971). The value for k_{12} is small when compared with a value at least 10 times as large, which is expected for a diffusion controlled process (Eigen and Hammes, 1963; Havsteen, 1969). The finding suggests that the observed reaction is not a simple, bimolecular process. The experiment was repeated at 13°C, and the plot of k_{obs} against concentration of L-isoleucine is presented in Figure 2b. The stronger fluorescence at lower temperature allowed us to extend our measurements up to 0.59 mM L-isoleucine. It is seen that the plot is no longer linear, but k_{obs} tends to become independent as concentration increases. The type of curve is consistent with the two-step scheme shown in equation (14) (Czerlinski, 1966).

$$E + Ile \quad \underset{}{\overset{K_{(1)}}{\rightleftharpoons}} \quad E\cdot Ile(1) \quad \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} \quad E\cdot Ile(2) \tag{14}$$

The first step of the process is fast and always in preequilibrium. Values for the observed rate constant follow expression (15)(Czerlinski, 1966):

$$k_{obs} = k_{32} + k_{23} \frac{(Ile)_o}{K_{(1)} + (Ile)_o} \tag{15}$$

where the dissociation constant is defined as $K_{(1)} = (E)\cdot(Ile)/(E\cdot Ile(1))$. Evaluation of the kinetic parameters was accomplished as has been described previously (Holler, Rupley and Hess, 1969 and 1970). The overall dissociation constant was calculated according to equation (16) (Hammes, Porter and Stark, 1971):

$$\frac{1}{K_{Ile}} = \frac{1}{K_{(1)}} \left(1 + \frac{k_{23}}{k_{32}}\right)$$

Results are listed in Table I. Again, the value calculated for

K_{Ile} is in agreement with the value obtained from titration (at 10°C). Our experimental value here is smaller than the one reported recently (Holler, Bennett and Calvin, 1971), because we have averaged over a larger number of values, including those from new experiments.

We have determined the contribution of the overall signal of the observed reaction to the magnitude of the fluorescence quenching evaluated at equilibrium, using saturating concentration of L-isoleucine (0.325 mM). We found that 95% of the equilibrium signal was generated during the observed process, clearly indicating that most, if not all, of the signal belongs to the second step of scheme (14) [since $(Ile)_o > K_{(1)}$ and the first step is in preequilibrium].

The effect of other ligands on the L-isoleucine binding kinetics was examined for solutions of L-isoleucine containing either ATP, $MgCl_2$, pyrophosphate plus $MgCl_2$or pyrophosphate plus $MgCl_2$ and NaCl. Neither of these compounds had any considerable effect as can be seen from Figure 2b.

ATP. Experiments were conducted at 13°C as reported for L-isoleucine. Because of light absorbance by ATP, the reaction signal was much weaker than observed for L-isoleucine. A plot of k_{obs} against $(ATP)_o$, Figure 3, indicated again a two-step process. The kinetic parameters were evaluated as described and are presented in Table I. The inset in Figure 3 illustrates the linearization procedure principally used (Holler, Rupley and Hess, 1969, 1970).

Contrary to the invariance of the kinetics for L-isoleucine against Mg^{2+}, we find that the kinetics for ATP are accelerated beyond the limit of the stopped-flow apparatus when 10 mM ATP plus 10 mM $MgCl_2$ and 1 mM

ATP plus 1 mM $MgCl_2$, respectively, were preincubated before rapid mixing with the enzyme.

The effect of L-isoleucine is not clear because an extremely small signal is obtained. It seems as if the ATP reaction is still observable; however, further experiments are needed.

Pyrophosphate. Pyrophosphate alone (0.09 mM to 5 mM) or pyrophosphate (2.4 mM) plus $MgCl_2$ (2.4 mM), when rapidly mixed with the enzyme, did not generate any time dependent process. We compared fluorescence intensity of the reaction mixture (2.4 mM pyrophosphate plus 2.4 mM $MgCl_2$) with the intensity for the 1:1 mixtures of enzyme and pyrophosphate plus $MgCl_2$ with buffer. Since the level of fluorescence was definitely lower for the reaction mixture (after correction for background), we concluded that equilibrium is attained within the mixing time of the stopped-flow apparatus. We have calculated the lower limits of the rate constants for pyrophosphate and the magnesium salts of pyrophosphate and ATP, and listed them in Table I.

In conclusion we can summarize the following important features:

1. ATP, in the presence of Mg^{2+}, and pyrophosphate bind much faster to Ile-tRNA synthetase than L-isoleucine.

2. In the absence of Mg^{2+} ions, ATP and L-isoleucine react at a similar slow rate, whereas pyrophosphate still reacts too fast to be measured.

3. The kinetics for ATP, but not for L-isoleucine, are drastically accelerated upon addition of Mg^{2+} ions.

Binding of ligands at catalytic condition. When Ile-tRNA synthetase was rapidly mixed with a solution containing ATP, L-isoleucine and Mg^{2+} ions, a second process appeared which was associated with fluorescence quenching, Figure 4a. This process was slower than the binding process

for L-isoleucine when the concentration of reactants was low [$(Ile)_o < K_{Ile}$, $(ATP)_o < K_{ATP}$] but became compatible at saturation concentration. We have evidence that the newly observed reaction can be ascribed to the formation of enzyme bound L-isoleucyl adenylate.

The next step in our investigation would have been the investigation of the binding kinetics for the various reactants of the amino acid activation reaction at catalytic condition. However, because of the limitation of our method, we can only make some general comments: (1) We observed one process at dilute concentrations of reactants, two processes at moderate concentrations [$(Ile)_o \sim K_{Ile}$, $(ATP)_o \sim K_{ATP}$] and one process at high concentrations of substrates [$(Ile)_o > K_{Ile}$, $(ATP)_o > K_{ATP}$]. The pattern is consistent with predictions from the results at non-catalytic condition, that the kinetics for L-isoleucine, but not for ATP (and PP_i) can be resolved. (2) The assumption that the kinetics for L-isoleucine were the same as at non-catalytic conditions proved to be valid, as will be shown for conditions when enzyme and saturating amounts of ATP were preincubated before mixing. (3) The values for the dissociation constants derived at ATP-[^{32}P]PP_i exchange conditions (Cole and Schimmel, 1970) are similar to those from titration experiments at non-catalytic conditions.

It appeared from these arguments that equation (9) could be applied, provided the formation of the enzyme bound L-isoleucyl adenylate was distinguishably slower than the binding process for L-isoleucine.

Formation of enzyme-L-isoleucyl~AMP complex at low concentrations of ligands [$(ATP)_o < K_{ATP}$, $(Ile)_o < K_{Ile}$, $(PP_i)_o < K_{pp}$]. When solutions of enzyme and reactants were rapidly mixed a slow quenching process was observed, Figure 4a. Initial concentrations (after mixing) were 0.075 μM

Ile-tRNA synthetase, 0.8 μM L-isoleucine, 11 μM ATP, 0.97 mM $MgCl_2$ and 43 μM TNS. The process was ascribed to the formation of enzyme bound L-isoleucyl adenylate on basis of the following arguments: (1) The quenching process was not observed for comparable concentrations of reactants at non-catalytic condition. (2) Berg (1958) has promoted the idea that the amino acid activation reaction is associated with the formation of an amino-acyl adenylate and isolation of enzyme bound L-isoleucyl∿AMP has been demonstrated (Norris and Berg, 1964; Eldred and Schimmel, 1972). (3) According to the equilibrium constants which have been reported by Cole and Schimmel (1970), the enzyme-L-isoleucyl ∿AMP complex had to be the only complex favored under the condition of our experiment. (4) As we shall demonstrate below, addition of pyrophosphate to the solutions of reactants enhanced the rate of the observed process and simultaneously decreased the overall signal. This observation was predicted from equation (9) and on basis of equilibrium calculations. (5) The rate of formation depended on Mg^{2+} ion concentration follows the same pK_{MgATP} (Holler and Calvin, manuscript in preparation) as has been shown by Cole and Schimmel (1970) to describe the formation of the only reactive species $MgATP^{2-}$

At dilute concentrations of reactants, the process of formation of enzyme-L-isoleucyl∿AMP complex was distinguishably slower than the process for binding of L-isoleucine to the enzyme, and we based our evaluations on equation (9). The expression for k_{obs} could be simplified, since $(ATP)_o \ll K_{ATP}$, $(Ile)_o \ll K_{Ile}$ and $(PP_i)_o \ll K_{pp}$, and we obtained the following equation:

$$k_{obs} = k_f \frac{(Ile)_o (ATP)_o}{K_{Ile} \cdot K_{ATP}} + k_b \frac{(PP_i)_o}{K_{PP}} \qquad (17)$$

Values of k_{obs} were measured for various concentrations of pyrophosphate at a fixed concentration of L-isoleucine and ATP. The kinetic parameters were then evaluated on basis of equation (17) from slope and intercept of the linear plots in Figure 5. The evaluated data are listed in Table II. Also listed are values calculated for the equilibrium constant of formation of the enzyme-L-isoleucyl AMP∿complex from substrates according to the following equation:

$$K_{eq} = \frac{(E)\cdot(ATP)\cdot(Ile)}{(E\cdot Ile\sim AMP)\cdot(PP_i)} = \frac{k_b}{k_f}\cdot\frac{K_{Ile}\cdot K_{ATP}}{K_{PP}} = \frac{\text{slope (Figure 5)}}{\text{intercept (Figure 5)}}\ (Ile)_o\cdot(ATP)_o \qquad (18)$$

For comparison, we also determined the value of K_{eq} at equilibrium conditions, using the stopped-flow apparatus (Figure 6) and fluorescence spectrophotometer (Figure 7). Evaluation was processed for dilute solutions when $(ATP)_o < K_{ATP}$, $(Ile)_o < K_{Ile}$ and $(PP)_o < K_{PP}$ and the formation of binary enzyme-ligand complexes is negligible. Evaluation was based on equation (18), which had been rearranged to the linear relations (19) and (20).

$$\Delta F = f_c\cdot(E)_o - K_{eq}\ (PP)_o\ \frac{F}{(Ile)_o\cdot(ATP)_o} \qquad (19)$$

$$\frac{1}{\Delta F} = \frac{1}{f_c\cdot(E)_o} + \frac{K_{eq}}{f_c\cdot(E)_o\cdot(Ile)_o\cdot(ATP)_o}\ (PP_i)_o \qquad (20)$$

where $\Delta F = f_c\cdot(E\cdot Ile\sim AMP)$. Equation (19) and equation (20) were applied when concentration of substrates and pyrophosphate, respectively, were varied. From comparison in Table II it is seen that the kinetically determined values for K_{eq} agree reasonably with the values from the equilibrium measurements.

From Figures 5 and 6 it is clear that addition of pyrophosphate to the substrate mixture enhances k_{obs} and reduces the overall change of fluorescence intensity as has been predicted. In addition, we predicted that rapid mixing of a solution containing enzyme, substrates and Mg^{2+} ions with a solution of pyrophosphate should be followed by a fluorescence enhancement, since the enzyme-bound L-isoleucyl adenylate which had been formed during preincubation would react with pyrophosphate to form free enzyme and substrates. Figure 4b shows the oscilloscope display for such an experiment. In agreement with the prediction, fluorescence intensity is enhanced at a rate with $k_{obs} = (17 \pm 1.2)\ sec^{-1}$. Interestingly, the value is close to 15 sec^{-1}, indicated by the broken line in Figure 5, which is the value determined for the rate constant k_{21} for dissociation of L-isoleucine from the enzyme-substrate complex, Table I. In agreement with our kinetic model, at conditions of increasing concentrations of pyrophosphate the dissociation of L-isoleucine becomes rate-limiting. $MgATP^{2-}$ dissociates much faster than L-isoleucine.

Formation of enzyme-L-isoleucyl∿AMP complex at high concentrations of ligands. The experiments were undertaken because of the following reasons: (1) They provide another independent measurement for the rate constant k_f. (2) They add proof that the dissociation constants measured at non-catalytic conditions are almost identical with those at catalytic conditions. (3) They may provide additional arguments about the kinetic pathway of the catalytic reaction.

We restricted our investigation so as to measure only reaction of formation of the enzyme-L-isoleucyl∿AMP complex. Since we chose dilute enzyme concentrations, those terms in equations (9) and (11) could be

dropped which contained the concentration of pyrophosphate. The validity of the simplification was tested experimentally by comparing the intercepts of the linear plots in Figure 5 with the values for k_{obs} which had been obtained from experiments where pyrophosphate had been omitted. At 1 mM $MgCl_2$ the value from the intercept is 0.9 sec^{-1} and at 8 mM $MgCl_2$, 0.3 sec^{-1}. The values are in agreement with (0.94 $\pm$ 0.13) sec^{-1} and (0.49 $\pm$ 0.02) sec^{-1}, respectively, which have been measured directly.

Under the conditions of very dilute concentration of pyrophosphate the expression (9) for the rate constant k_{obs} was reduced to

$$k_{obs} = k_f \frac{(Ile)_o}{K_{Ile}+(Ile)_o} \cdot \frac{(ATP)_o}{K_{ATP}+(ATP)_o} \qquad (21)$$

Enzyme plus L-isoleucine were preincubated at saturation concentration, and then rapidly mixed with a solution containing a mixture of ATP at rising concentrations, and 8 mM $MgCl_2$. The interpretation was not complicated by the slow L-isoleucine binding process. Figure 8a presents a plot of k_{obs} as a function of the concentration of ATP. The type of concentration dependence is hyperbolic as predicted from equation (21) for a fixed concentration of L-isoleucine. It is immediately evident that k_{obs} approaches k_f because L-isoleucine is saturating. Applying the usual procedures, values for k_f and K_{ATP} were determined from the linear plot, Figure 8a, inset (Table III). Similarly, enzyme was preincubated with ATP at saturating concentration and was then rapidly mixed with a solution containing $MgCl_2$ and L-isoleucine at varying concentrations. For this experiment, the kinetics for binding of L-isoleucine were expected to interfere with the kinetics for the formation of enzyme-L-isoleucyl~AMP and the steady-state equation (11) had to be applied. The value for $(k_{21}+k_f)/k_{12}$ was determined from the slope of the linear plot in Figure 8b, inset. The importance of

the steady-state approach is recognized, when the value 68 μM from the slope is compared with the value 5.8 μM for K_{Ile} as expected if the rate of dissociation of L-isoleucine from the ternary enzyme-substrate complex would have been fast ($k_{21} >> k_f$). Applying the known values for K_{Ile} and k_{12}, we calculated the value 135 sec^{-1} for k_f which is in agreement with the value 105 sec^{-1} obtained from the intercept of the same linear plot (Figure 8b). In conclusion, the agreement for kinetic parameters and equilibrium constants at conditions of low and high concentrations of reactants (Tables II and III), indicates that our extrapolations from experiments at non-catalytic conditions were valid.

Effect of temperature. We have determined the dissociation constants for formation of binary enzyme-ligand complexes for L-isoleucine and ATP in the temperature range between 10°C and 37°C, and for pyrophosphate at 10°C and 25°C using the titration method. Thermodynamic parameters were determined from Van't Hoff-plots, Figure 9. The temperature dependence of the amino acid activation reaction was measured in terms of the slopes and intercepts of Figure 5 in the range from 13°C to 37°C. Values for the thermodynamic activation parameters were determined from Arrhenius plots (Glasstone, Laider and Eyring, 1941). Results are listed in Table IV and Table V, respectively.

Discussion

Binding of L-isoleucine, ATP and pyrophosphate. The interaction of L-isoleucine and ATP. respectively, with Ile-tRNA synthetase does not follow simple bimolecular kinetics, but presumably a two-step mechanism where the second process is an isomerization of the first enzyme-substrate

complex. A substrate induced conformation change has been previously assumed to be associated with the varying degree of energy transfer from the protein to TNS (Holler, Bennett and Calvin, 1971) and with the acceleration of the association-dissociation reaction for Ile-tRNA^{Ile} in the presence of substrates (Yarus and Berg, 1969; Eldred and Schimmel, Biochemistry, in press). Substrate induced protein isomerization steps have been observed in lysozyme-catalyzed reactions (Holler, Rupley and Hess, 1969 and 1970), in ribonuclease-catalyzed reactions (French and Hammes, 1965) and in reactions catalyzed by a number of other enzymes (Hammes, 1968; Gutfreund, 1971).

The reaction for L-isoleucine appears to become bimolecular when the temperature is raised to 25°C, however, the rate of the association reaction being too slow to be a diffusion controlled process. A two-step process seems to be of the one-step type when $K_{(1)} >> (Ile)_o$ in equation (15). Then the bimolecular rate constants will be interpreted as $k_{12} = k_{23}/K_{(1)}$ and $k_{21} = k_{32}$. From the data in Figure 2b we find that $k_{23} > 400$ sec^{-1} and $K_{(1)} > 0.2$ mM. The data for the two-step process at 13° and 25°C indicate that the first enzyme L-isoleucine complex tends to become unstable at higher temperature, whereas the reverse is true for the second complex. Such a tendency is compatible with hydrogen bonding and electrostatic forces stabilizing the first complex and hydrophobic interactions stabilizing the second complex (Kauzman, 1959). Furthermore, the involvement of hydrophobic interactions is evident for the following reasons: 1. The specific side chain of L-isoleucine is hydrophobic. 2. The standard enthalpy of the formation of the enzyme-L-isoleucine complex is slightly positive as determined from titration experiments. 3. The

standard entropy is positive as expected for H_2O-structure decreasing interactions (Nemethy and Scheraga, 1963; Nemethy, Steinberg and Scheraga, 1963; Heidberg, Holler and Hartmann, 1967).

The kinetics for L-isoleucine are not changed when Mg^{2+} ions are present, and not even under catalytic conditions. Evidence is clearly provided by the experiment shown in Figure 8b when a solution of L-isoleucine and $MgCl_2$ is rapidly mixed with a solution of enzyme saturated previously with ATP (the assumption is made that Mg^{2+} combines rapidly with ATP). The dependence of k_{obs} on the concentration of L-isoleucine is in agreement with the rate constants evaluated under non-catalytic conditions (Figure 2). In addition, the values for K_{Ile} which have been determined under catalytic condition via the ATP-[^{32}P]PP_i exchange assay are in close agreement with our titration data (Cole and Schimmel, 1970).

In contrast, the kinetics for ATP binding are drastically accelerated in the presence of Mg^{2+} ions and are beyond the limit of stopped-flow measurements. The same seems to be true at catalytic conditions when Mg^{2+} ions plus L-isoleucine are present. Although the kinetics are drastically changed, the dissociation constant K_{ATP} remains unchanged when the reactants are added one by one. This is seen for the absence and presence of Mg^{2+} ions or L-isoleucine (Holler, Bennett and Calvin, 1971) and the presence of both as shown by the experiments in Figure 8a. This somewhat surprising observation reminds us of the finding by Yarus and Berg (1969) that binding of L-isoleucine promotes the kinetics of Ile-$tRNA^{Ile}$ binding without changing the equilibrium constant.

The kinetics for pyrophosphate were beyond the stopped-flow limit at all conditions tested. The experimental results obtained from titration

experiments (Holler, Bennett and Calvin, 1971) and ATP-[^{32}P]PP_i exchange measurements (Cole and Schimmel, 1970) indicate that pyrophosphate binds to Ile-tRNA synthetase with an affinity almost identical with that for ATP. When we reexamined the value for the dissociation constant at 10°C we found that even the temperature dependence resembled closely that for ATP.

In summary, we have strong evidence that our evaluation is based on the valid assumption that the dissociation-association equilibria of the various ligands as described by their dissociation constants are identical under a variety of compositions of the reaction mixture.

Kinetics of the isoleucine-activation step. The rate constant for the transfer of the AMP-moiety from pyrophosphate to L-isoleucine when bound to Ile-tRNA synthetase is found to be 135 sec^{-1} (1 mM $MgCl_2$) and 670 sec^{-1} for the reverse process. The value of the equilibrium constant described as $(E \cdot Ile \sim AMP \cdot PP)/(E \cdot Ile \cdot ATP) = k_f/k_b$ is 0.2. The equilibrium is not on the side of the enzyme-product complex. This is not surprising in face of the known standard free enthalpy of hydrolysis for ATP and acetyl∿AMP, the acetyl∿AMP being more labile by 5.7 kcal (pH 7.0) than ATP (Mahler and Cordes, 1968). Thus, free amino acyl∿AMP has a free energy content which is approximately 6 kcal higher than for ATP (no corrections were made to compensate for pH effects). Yet, this difference is reduced to less than 1 kcal when the reactants are placed into enzymic environment. In decreasing the difference in free energy content by complex formation we would have a free energy trap which is deeper for the products than for ATP plus L-isoleucine. One can imagine that leveling of the standard free enthalpy could also

result in a considerable decrease of the free energy of the transition state, thus providing an enzymic mechanism for rate enhancement.

The dissociation constant for L-isoleucyl∿AMP is calculated from the overall equilibrium constant of the amino acid activation reaction, K_{over}, and from K_{eq}. The value for K_{eq} has been determined in Table II to be in the range 8 μM to 28 μM (1 mM $MgCl_2$). A recently reported value of 7 μM is in agreement with our data (Penzer, Bennett and Calvin, 1970). The value 0.33 μM reported from ATP-[^{32}P]PP_i-exchange investigation (Cole and Schimmel, 1970) may be lower because of Mg^{2+} ion and ionic strength effects. An explanation has been offered by Penzer, Bennett and Calvin (1970). The dissociation constant for the enzyme-L-isoleucyl ∿AMP complex, $K_{Ile\ AMP}$, is calculated with use of the following relation

$$K_{over} = \frac{(PP)\cdot(Ile\sim AMP)}{(Ile)\cdot(ATP)} = \frac{K_{Ile\sim AMP}}{K_{eq}} = 4.5\ 10^{-4}$$

The calculated value for $K_{Ile\sim AMP}$ falls into the range 4 nM to 12 nM. The value is found to be in close agreement with the dissociation constant 13 nm (10°C) for binding of the analog L-isoleucinol-AMP to Ile-tRNA synthetase (Holler et al., manuscript in preparation).

We found that the rate of cleavage of enzyme bound L-isoleucyl∿AMP into free substrates does not exceed the rate of dissociation of L-isoleucine from the enzyme substrate complex. This is because the rate constant for dissociation, $k_{21} = 15\ sec^{-1}$ is smaller than the rate constants, k_f, k_b, of the amino acid activation step. However, the relation may not be important in the case of ATP-[^{32}P]PP_i-exchange measurements since, according to our results, the dissociation of ATP and thus exchange at the level of the Michaelis-Menten complex would be very fast.

Order of substrate addition. According to what we have mentioned in the derivation of the kinetic expressions, it is not possible to distinguish whether the sequence of reaction (3) is ordered or random, when there are rapid preequilibria of all the association-dissociation reactions and because of the insensitivity of dissociation constants against co-ligands. However, in case we would have an unproductive ternary enzyme-substrate complex we should evaluate k_f at dilute solutions of substrates, $(Ile)_o < K_{Ile}$, $(ATP)_o < K_{ATP}$, to be about twice the value at saturation conditions. That our system does not follow this prediction is seen from comparison of the data in Table II and Table III. However, because of the considerable experimental error we should not take the result too seriously.

Evidence that L-isoleucine would not bind second is obtained by taking advantage of the slowness of the L-isoleucine dissociation reaction. We chose a condition which is not compatible with rapid pre-equilibrium, by preincubating enzyme plus saturating amounts of L-isoleucine, Figure 8a. If the amino acid does not bind first, it would have to dissociate so that ATP could bind first. Since the rate of dissociation is slow, we would expect k_{obs} not to exceed the value 15 sec^{-1}. The prediction is clearly not fulfilled, indicating that L-isoleucine either binds first or in a random sequence. We have restricted, so far, the types of possible kinetic mechanisms; however, we cannot conclusively say whether we have a random or ordered pathway. We believe that the invariance of the dissociation constants against binding of co-ligands could be best understood for a random mechanism. The types of kinetic mechanisms which have so far been reported for various aminoacyl-tRNA

synthetases have been recently reviewed by Santi, Danenberg and Satterly (1971).

Effect of temperature. The temperature dependence of the various kinetic and equilibrium constants as depicted in Figures 9, a and b, exhibits no discontinuity. The observed linear relations are in accord with the assumption of only one type of active site (Dawes, 1964). Analysis of temperature effects in enzymic systems is subject to controversial opinions. As in most cases, the observed thermodynamic parameters cannot be broken down to the level of elementary events. Only in some cases, as is seen for hydrophobic interactions, can the observed values be ascribed convincingly to a certain type of interaction. Along this line it is difficult to interpret the thermodynamic parameters for ATP and pyrophosphate binding. From the fact that standard enthalpy and entropy are both negative, one would like to conclude that several hydrogen-bonds are involved in holding the enzyme-ligand complexes together (Kauzmann, 1959). However, since both ligands are highly negatively charged, one would also predict pure electrostatic interactions.

We have attempted to obtain information about the thermodynamic parameters which govern the catalytic reaction. We have calculated the values for the activation enthalpies and the standard enthalpy of formation of enzyme-bound products as described in Table VI. We have tentatively used the standard enthalpies for enzyme-reactant complex formation as measured by titration experiments at non-catalytic conditions. For this reason we do not expect our calculation to give exact results. However, since our investigation at 25°C has shown that the dissociation constants are very similar for catalytic and non-catalytic conditions we consider the data as a sensible approach. The results are interesting

from a qualitative point of view. The activation enthalpies have moderate values as might be expected for an enzyme catalyzed reaction. The reaction enthalpy $\Delta H^{o}(k_f/k_b)$ is small, providing an almost temperature independent equilibrium constant. Thus, as temperature is raised from 25°C to 37°C the value for k_f/k_b = $(E \cdot Ile \sim AMP \cdot PP_i)/(E \cdot Ile \cdot ATP)$ increases only slightly, from 0.20 to 0.28.

When the thermodynamic standard parameters are small one may conclude that the enzyme-bound substrates are virtually enzyme-bound products, supporting the idea that the ternary enzyme-substrate complex might be of a configuration half way to the transition state. Free enthalpy considerations suggest that in particular ATP, when bound, exists in a high free energy state (Holler et al., manuscript in preparation).

We have presented kinetic and thermodynamic features of the isoleucine activating enzyme system. We are confident that some of the results, such as substrate induced conformation changes, Mg^{2+} ion effects and equilibrium concentrations of the ternary enzyme-substrate and enzyme-product complexes are of common interest for a number of aminoacyl-tRNA synthetases. The interesting question which has to be answered next is how binding of a cognate tRNA will modify the kinetic and equilibrium parameters.

We are very grateful to Professor Jack F. Kirsch, Department of Biochemistry, University of California, Berkeley, who kindly allowed us to use his stopped-flow apparatus. We thank Dr. Edward L. Bennett for his stimulating discussions and encouragement, and Mrs. Ann Orme for her invaluable help during isolation of the enzyme.

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TABLE I: Formation of binary Ile-tRNA synthetase-reactant complexes at pH 8.0.[a]

Kinetic parameters[b] and equilibrium constants.[c]

Reactant	T(°C)	buffer	one-step process k_{12} ($M^{-1}sec^{-1}$)	one-step process k_{21} (sec^{-1})	two-step process k_{23} (sec^{-1})	two-step process k_{32} (sec^{-1})	$K_{(1)}$ (mM)	overall dissociation constant calc. (μM)	overall dissociation constant obs. (μM)
Ile	25	P or T	$2.2 \cdot 10^6$	15	--	--	--	6.8	5.8 ± 0.8
	13	P or T	--	--	125	3	0.093	2.2	
	10	P or T							6.0 ± 1.6
ATP	13	P	--	--	30	4	4.0	500	
	10	P or T							140 ± 40
Mg ATP^{2-}[d]	25	T	> $2 \cdot 10^6$[e]		> 400[e]				175 ± 50
PP_i	25	P or T							260 ± 70
Mg PP_i^{2-}[d]	25	T							190 ± 50

[a] Initial concentrations were 0.05 μM to 0.2 μM Ile-tRNA synthetase, 1 μM to 50 μM TNS, 0.02 M sodium phosphate buffer (P), 0.05 M Tris-HCl buffer (T) and 0.01 M 2-mercaptoethanol.

[b] As defined by equations (12) and (14).

[c] Calculated according to equation (16) or determined by titration (Holler, Bennett and Calvin, 1971).

[d] Solutions contained ATP (or PP_i) and Mg^{2+} ions on a 1:1 concentration basis.

[e] Calculated, assuming the reaction is completed within 4 msec, the dead time of the stopped-flow apparatus.

TABLE II: Kinetic parameters of the L-isoleucine activation reaction at pH 8.0, 25°C[a]

Parameters	$MgCl_2$ 1 mM	$MgCl_2$ 8 mM
slope (10^6 $M^{-1}sec^{-1}$)	2.57	1.38
intercept (sec^{-1})	0.9	0.3
K_{Ile} (μM)	5.8	5.8
K_{ATP} (mM)	0.25	0.25
K_{PP} (mM)	0.26	0.26
k_f (sec^{-1})	135	45
k_b (sec^{-1})	670	360
	(685)	(1000)
k_f/k_b	0.20	0.12
	(0.20)	(0.045)
K_{eq} (μM)	28[b]	47[b]
	8.2[c]	5.7[d]
	22[f]	(16)[d]
		7.7[e]
		(21)[e]

TABLE II (Cont.)

[a]Parameters were evaluated from Figures 5 to 7 according to equations (17) to (20). The values for K_{Ile}, K_{ATP} and K_{PP} which were used for computation are indicated. Values in parentheses have been corrected for the formation of catalytically inactive species $Mg_2P_2O_7$ and $MgHP_2O_7$ (Cole and Schimmel, 1970). Correction factors used are 1.02 at 1 mM $MgCl_2$ and 2.78 at 8 mM $MgCl_2$.

[b]Calculated from kinetic parameters in this table.

[c]Determined from the overall fluorescence quenching of the reaction in Figure 6a as function of the concentration of pyrophosphate.

[d]Determined from the overall fluorescence quenching of the reaction in Figure 6b as function of the concentration of substrates.

[e]Determined from equilibirum measurements using a spectrofluorimeter and varying the concentration of L-isoleucine, Figure 7.

TABLE III: Kinetic parameters for the formation of Ile-tRNA synthetase-L-isoleucyl∿AMP complex at high concentration of substrates at pH 8.0, 25°C.[a]

Kinetic parameter	Ile-tRNA synthetase preincubated with	
	0.13 mM L-isoleucine	1 mM ATP
k_f (sec^{-1})	62[b]	105[b] 135[c]
K_{ATP} (mM)	0.173[d]	
K_{eq} (μM)[e]	32	19 15

[a]Experimental conditions were those of Figure 8. Evaluation has been based on equations (11) and (21).

[b]Rate constants were determined from the intercept of the linear plots in Figure 8 (insets).

[c]The rate constant has been determined from the slope of the line in Figure 8b, inset. The relation used is -slope = $K_{Ile} + k_f/k_{12}$. The values for k_{12} and K_{Ile} were taken from Tables I and II, respectively.

[d]As determined from the slope of the line in Figure 8a, inset.

[e]Calculated according to equation (18) with use of the values for the kinetic parameters (except for k_f) as given in Table II.

TABLE IV: Standard free energy, enthalpy and entropy for formation of binary Ile-tRNA synthetase-reactant complexes at pH 8.0.[a]

Reactant	Formation of binary enzyme-ligand complex		
	ΔG^o (kcal)	ΔH^o (kcal)	ΔS^o (e.u.)
L-isoleucine	-7.6	0.8	23
ATP	-5.0	-9.9	-15
Pyrophosphate	-5.0	-8.0	-10

[a]Evaluated from Figure 9a.

TABLE V: Standard free energy, enthalpy and entropy of activation for the kinetic parameters measured as slope and intercept in Figure 5.

Parameters, Figure 5[a]	Activation parameters $\Delta G^{o\ddagger}$ (kcal)	$\Delta H^{o\ddagger}$ (kcal)	$\Delta S^{o\ddagger}$ (e.u.)
Slope	9	14	16
Intercept	19	20	5

[a] 1 mM $MgCl_2$, evaluation of Figure 9b.

TABLE VI: Calculation of values for the standard reaction enthalpy and standard activation enthalpy of the L-isoleucine activation reaction,[a] pH 8.0.

$\Delta H^{o\ddagger}$ (k_f) = $\Delta H^{o\ddagger}$ (intercept) + ΔH^o (K_{Ile}) + ΔH^o (K_{ATP}) = 11 kcal
$\Delta H^{o\ddagger}$ (k_b) = $\Delta H^{o\ddagger}$ (slope) + ΔH^o (K_{pp}) = 6 kcal
ΔH^o (k_f/k_b) = 5 kcal

[a]Calculation is based on equation (17) and values in Tables IV and V.

FIGURE LEGENDS

Figure 1: Oscilloscope display for the formation of Ile-tRNA-L-isoleucine complex at pH 8.0, 13°C. The different traces are generated for repeated reactions. The reaction is started by mixing equal volumes of a solution containing 0.1 μM Ile-tRNA synthetase and of a solution containing 1.96 μM L-isoleucine. The solutions contained 0.05 M Tris-HCl buffer, 0.01 M 2-mercaptoethanol, and 43 μM TNS. Instrument settings were 1000 V photomultiplier, 2 mm slit for the excitation light beam, and 1 msec as the time constant of the electronic array for smoothing the signal from the photomultiplier.

Figure 2: Binding of L-isoleucine to Ile-tRNA synthetase at pH 8.0. Rate constants at 25°C (a) and 13°C (b). Initial concentrations (after mixing) were 0.075 μM to 0.12 μM Ile-tRNA synthetase, 43 μM TNS, 0.01 M 2-mercaptoethanol and 0.02 M sodium phosphate buffer (●) or 0.05 M Tris-HCl (□) buffer. k_{obs} was also determined in presence of 5.15 mM ATP (o), 2.4 mM $MgCl_2$ (■), 4.9 mM PP_i plus 5.3 mM $MgCl_2$ (▼), and 10 mM $MgCl_2$ plus 40 mM NaCl (▽). The data represent average values for three to six determinations of k_{obs}; the bars indicate mean deviations. The line in (a) has been computed on basis of equation (15) with use of the kinetic parameters presented in Table I, which have been evaluated for the data marked by filled circles.

Figure 3: Reaction rates for binding of ATP at non-catalytic conditions, pH 8.0, 13°C. Initial concentrations (after mixing) were 0.12 μM Ile-tRNA synthetase, 18 μM TNS, 0.02 M sodium phosphate buffer and 0.01 2-mercaptoethanol. Data represent average values for three or more determinations, the bars represent mean deviations. The linear plot in the inset is based on the relation $k_{obs}-k_{32} = k_{23}-K_{(1)}\cdot(k_{obs}-k_{32})/(ATP)_o$. The equation can be derived from the expression (15) for ATP. We have used the value $k_{32} = 4\ sec^{-1}$. The curve is calculated according to equation (15) (for ATP) assuming the values listed in Table I.

Figure 4: Oscilloscope displays (a) for the formation of Ile-tRNA synthetase-L-isoleucyl∿AMP complex after rapid mixing of a mixture of ATP, $MgCl_2$, and L-isoleucine with enzyme and (b) the reverse reaction after rapid mixing of an incubated mixture of ATP, $MgCl_2$, L-isoleucine and enzyme with pyrophosphate. Solutions contained 0.05 M Tris-HCl buffer, pH 8.0 (25°C) and 0.01 M 2-mercaptoethanol. Initial concentrations (after mixing) were 0.075 μM enzyme, 43 μM TNS, and 1 mM $MgCl_2$ in both experiments, 0.88 μM L-isoleucine and 11 μM ATP in experiment (a) and 1.47 μM L-isoleucine, 18.8 μM ATP and 55 μM pyrophosphate in experiment (b). The horizontal traces indicate levels of fluorescence after equilibrium had been established. Instrument settings were the same as in Figure 1 except that time constants of 10 msec and 5 msec, respectively, were used.

Figure 5: Rate constants for the formation of enzyme-L-isoleucyl∿AMP complex as function of initial concentration of pyrophosphate at pH 8.0, 25°C. Initial concentrations (after mixing) were 0.04 μM and 0.08 μM Ile-tRNA synthetase, 43 μM TNS, 0.87 μM L-isoleucine, 11 μM ATP, 0.05 M Tris-HCl buffer, 0.01 M 2-mercaptoethanol and (●) 1 mM $MgCl_2$ or (⊖) 8 mM $MgCl_2$. Slope and intercept of the solid lines are used for evaluation of kinetic parameter according to equation (17). The broken line determines the upper limit for k_{obs}, identical with the rate for dissociation of L-isoleucine from the enzyme-substrate complex.

Figure 6: Overall quenching of fluorescence after rapid mixing as function of concentration of pyrophosphate and substrates at catalytic conditions, pH 8.0, 25°C.

(a), concentration of pyrophosphate varied. Solutions are the same as for Figure 5 at 1 mM $MgCl_2$.

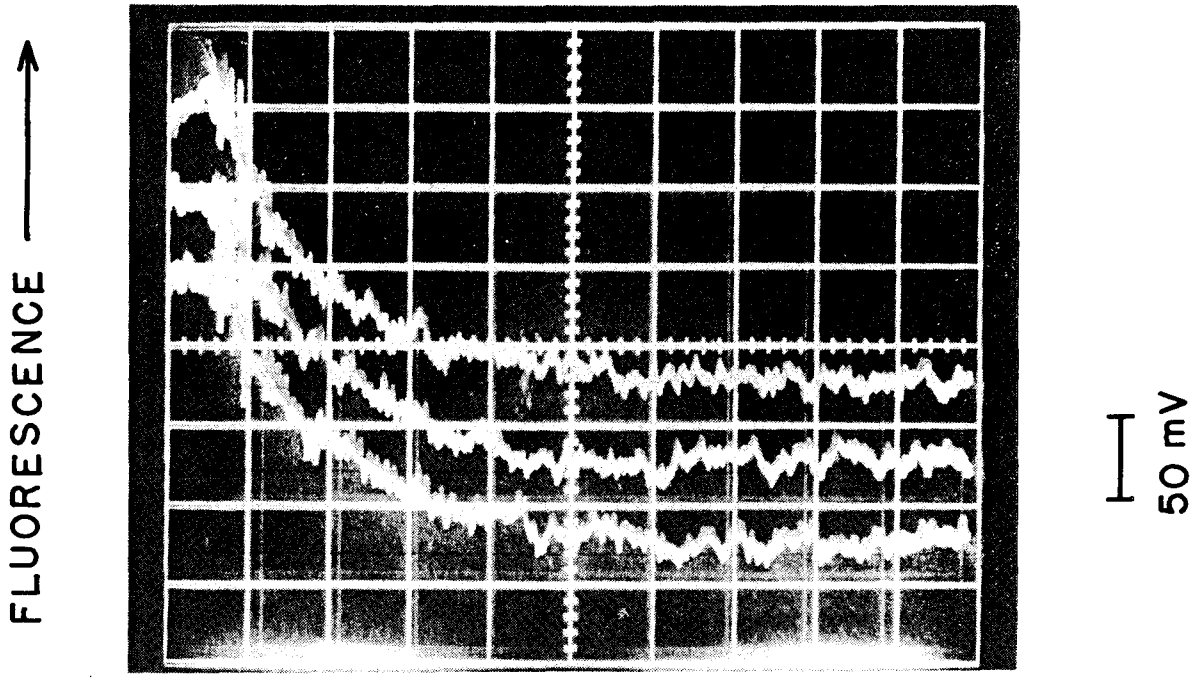
(b), concentration of L-isoleucine (o), or ATP (□) varied. Initial concentrations as in Figure 5 at 8 mM $MgCl_2$ except that $(PP_i)_o$ was constant 1.1 μM. Insets are plots according to equation (20) for (a) and according to equation (19) for (b), respectively. Data represent averages over three to five determinations, bars indicate mean deviations.

Figure 7: Fluorescence quenching as function of concentration of L-isoleucine observed with a spectrofluorimeter at catalytic conditions, pH 8.0, 25°C. Data are plotted according to equation (19) except that concentration of ATP was fixed. Initial concentrations were 0.19 μM Ile-tRNA synthetase, 14 μM ATP, 0.94 μM pyrophosphate, 1.14 mM $MgCl_2$, 12 μM TNS, 0.05 M Tris-HCl buffer, and 0.01 M 2-mercaptoethanol. Concentration of L-isoleucine was varied between 0.46 μM and 2.2 μM.

Figure 8: Rate constants for the formation of Ile-tRNA synthetase-L-isoleucyl~AMP complex at high concentrations of L-isoleucine and ATP, at pH 8.0, 25°C. Initial concentrations (after mixing) were 0.075 μM enzyme, 43 μM TNS, 8 mM $MgCl_2$, 0.05 M Tris-HCl buffer and 0.01 M 2-mercaptoethanol.

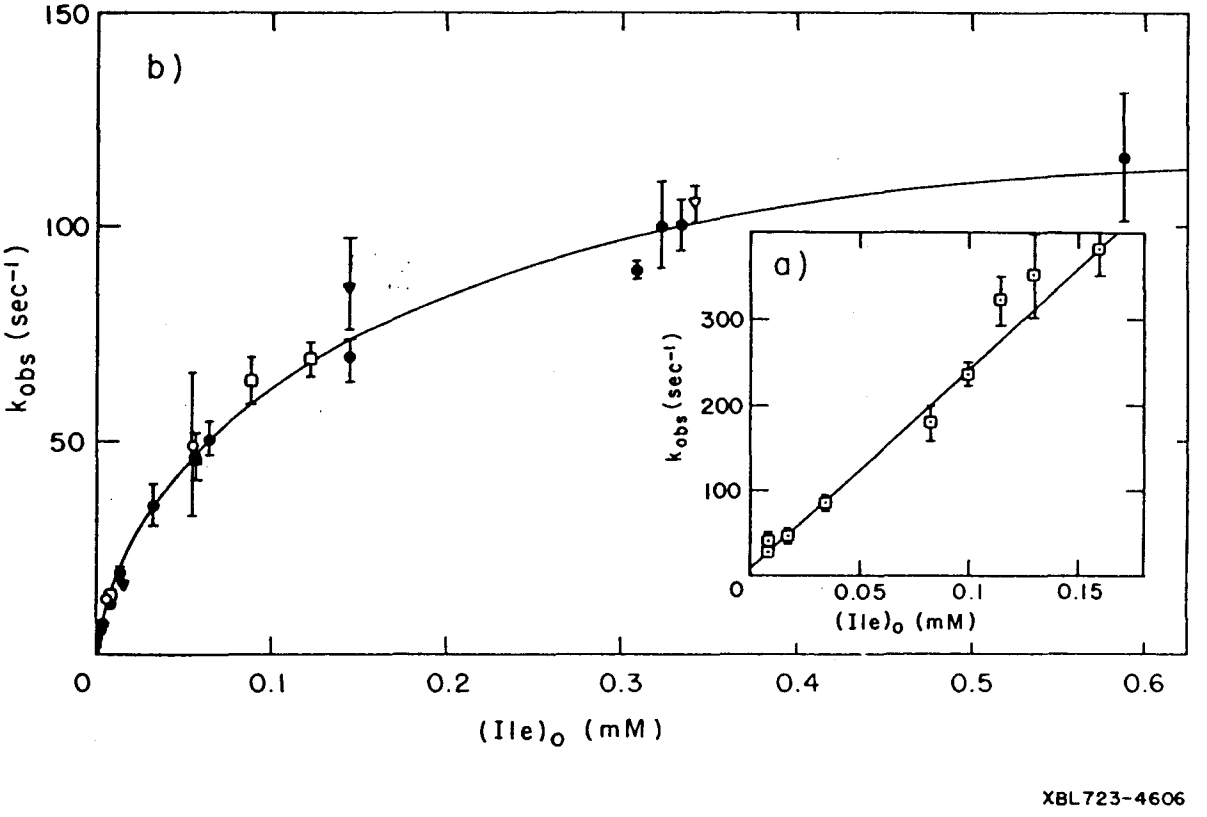
(a), enzyme was preincubated with 0.13 mM L-isoleucine (1 mM EDTA), and (b), with 1 mM ATP (1 mM EDTA). Data represent average values of three to five measurements, mean deviations are indicated by bars. Rate constants were replotted (insets) according to rearranged linear relations (Eadie, 1942) derived from equations (11) and (21). Kinetic parameters were evaluated from slopes and intercepts.

Figure 9: Temperature dependence (a) for dissociation constants of binary Ile-tRNA synthetase-reactant complexes, and (b) for kinetics of enzyme-L-isoleucyl∿AMP formation in terms of slope and intercept in Figure 5. Dissociation constants were determined from titration experiments in presence of 5 μM to 20 μM TNS (0.02 M sodium phosphate buffer or 0.05 M Tris-HCl buffer, pH 8.0, 0.01 M 2-mercaptoethanol) for L-isoleucine (Δ), ATP (□), and pyrophosphate (■). Part of the data has been published (Holler, Bennett, and Calvin, 1971). Kinetic constants are given as slope (●) and intercept (o) according to equation (17) and Figure 5. Initial concentrations were 1 mM $MgCl_2$ and otherwise the same as for Figure 5.



XBB 723-1577

Fig. 1



XBL723-4606

Fig. 2

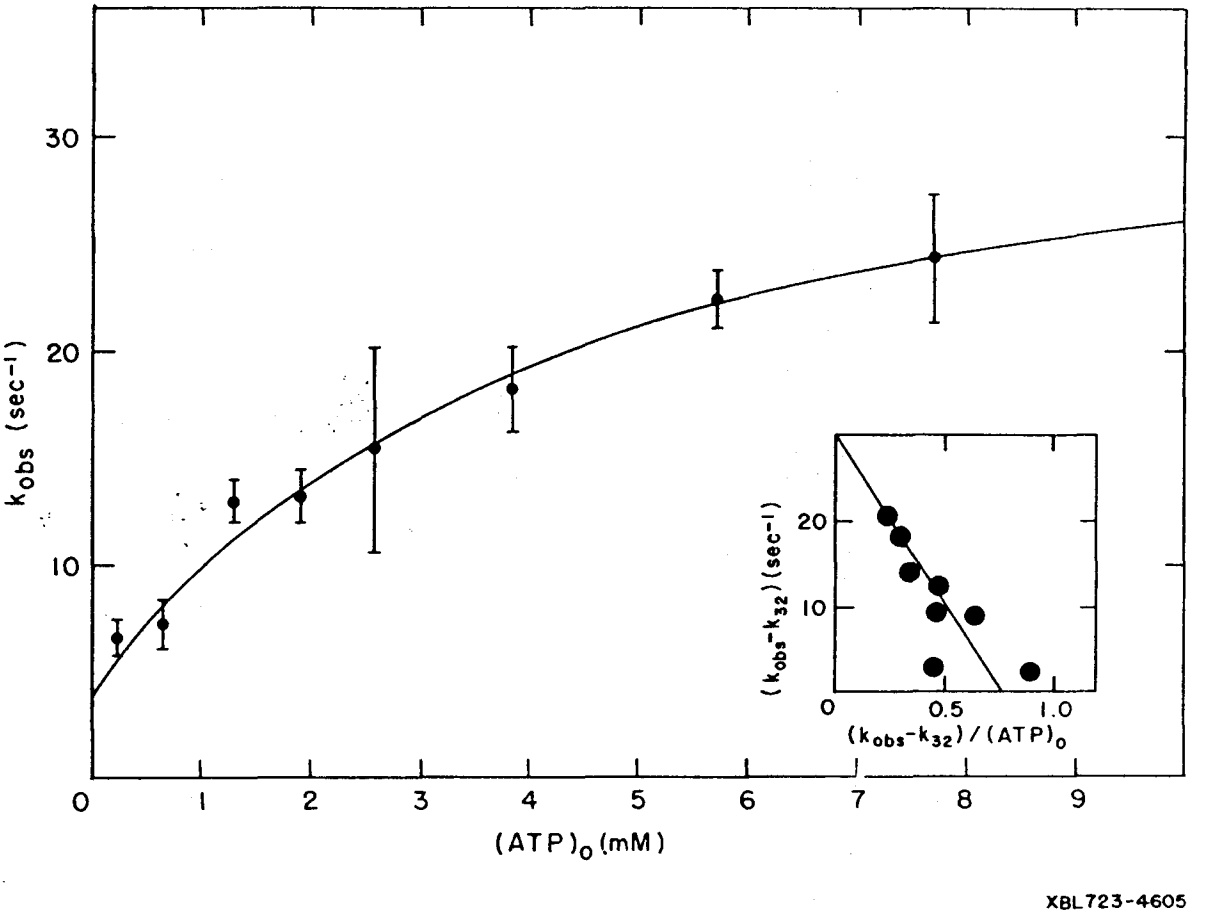

30
20
10
0
1
2
3
4
5
6
7
8
9
k_{obs} (sec^{-1})
$(ATP)_0$ (mM)
20
10
0
0.5
1.0
$(k_{obs}-k_{32})$ (sec^{-1})
$(k_{obs}-k_{32})/(ATP)_0$
XBL723-4605

Fig. 3

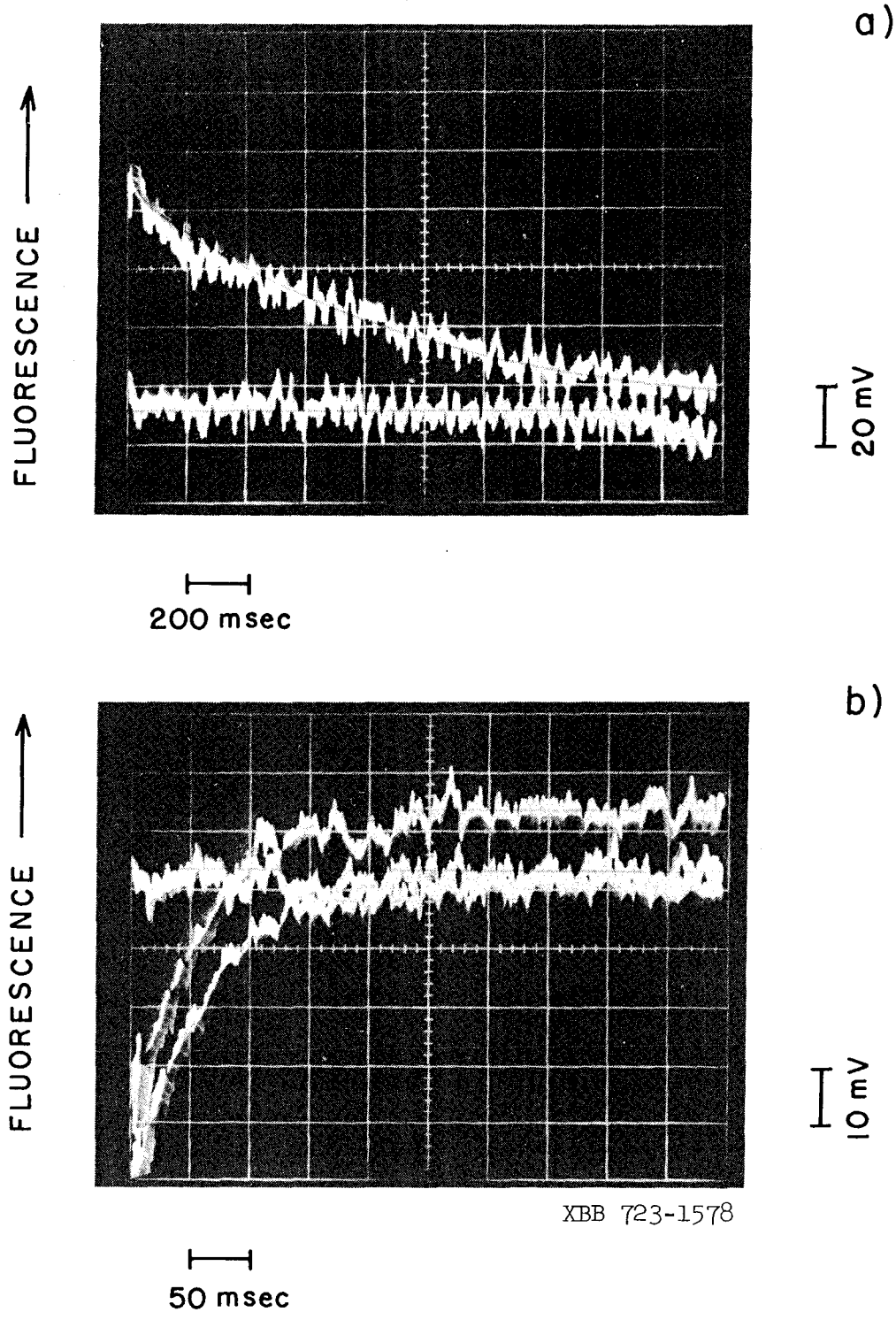

a)
FLUORESCENCE
20 mV
200 msec
b)
FLUORESCENCE
10 mV
XBB 723-1578
50 msec

Fig. 4

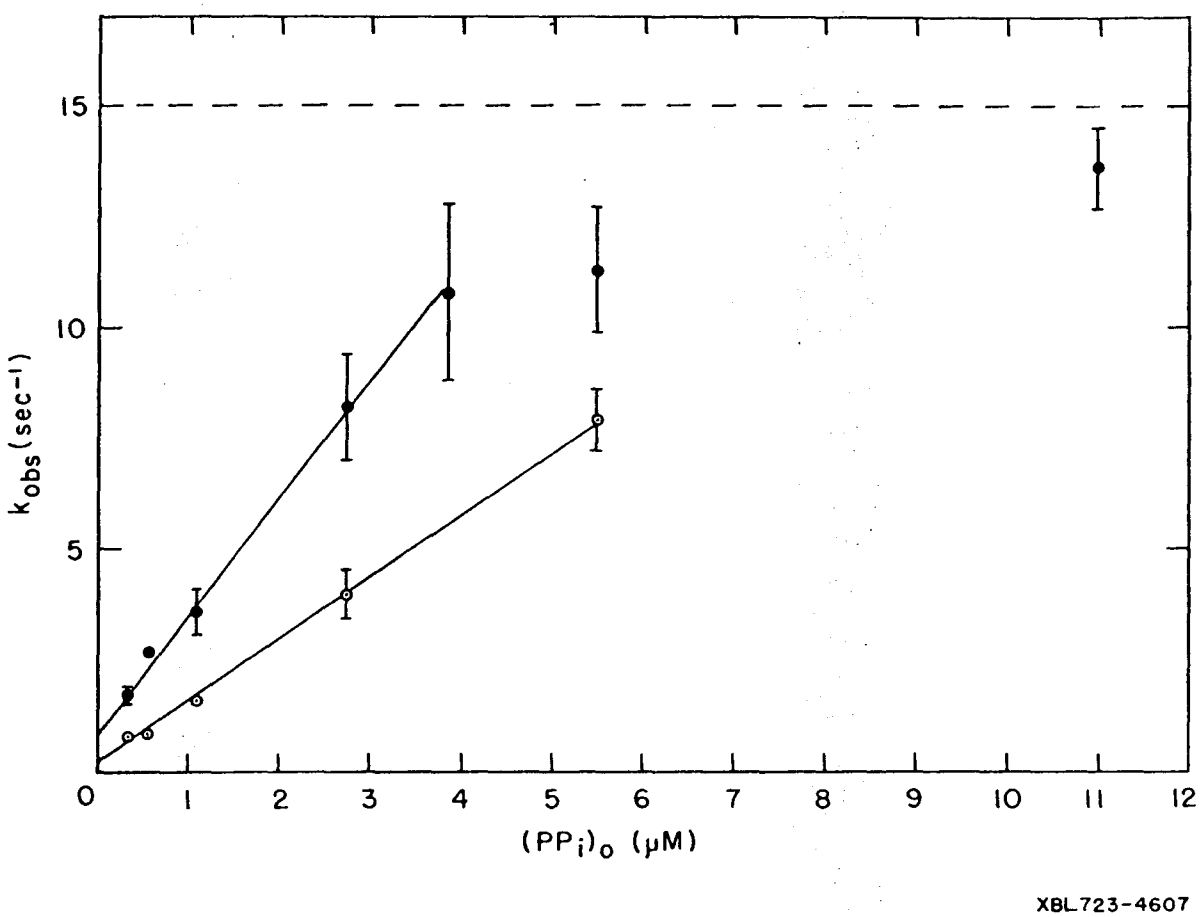


Fig. 5

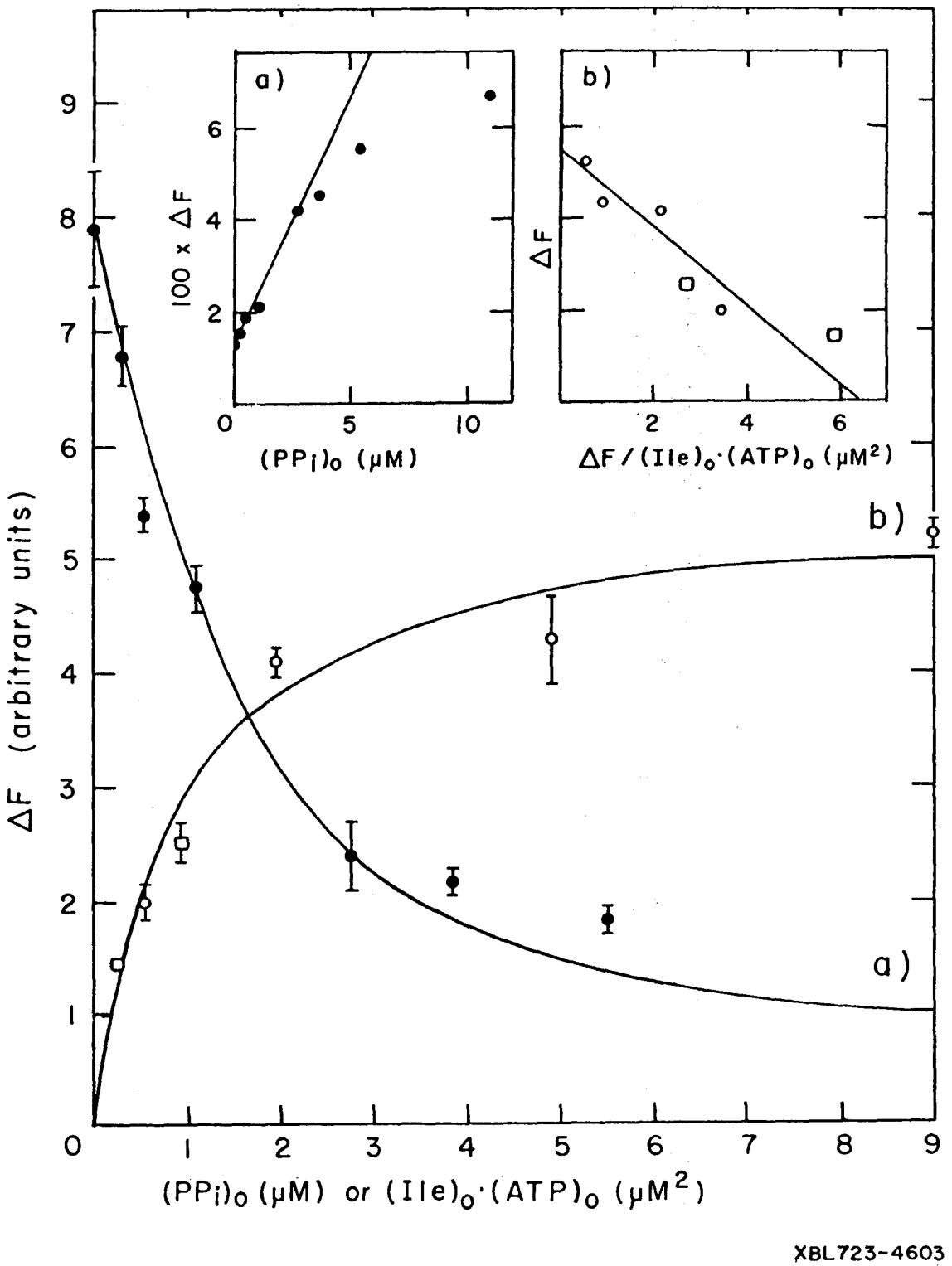

a)
b)
100 x ΔF
(PPi)o (μM)
ΔF
ΔF / (Ile)o·(ATP)o (μM²)
ΔF (arbitrary units)
(PPi)o (μM) or (Ile)o·(ATP)o (μM²)
XBL723-4603

Fig. 6

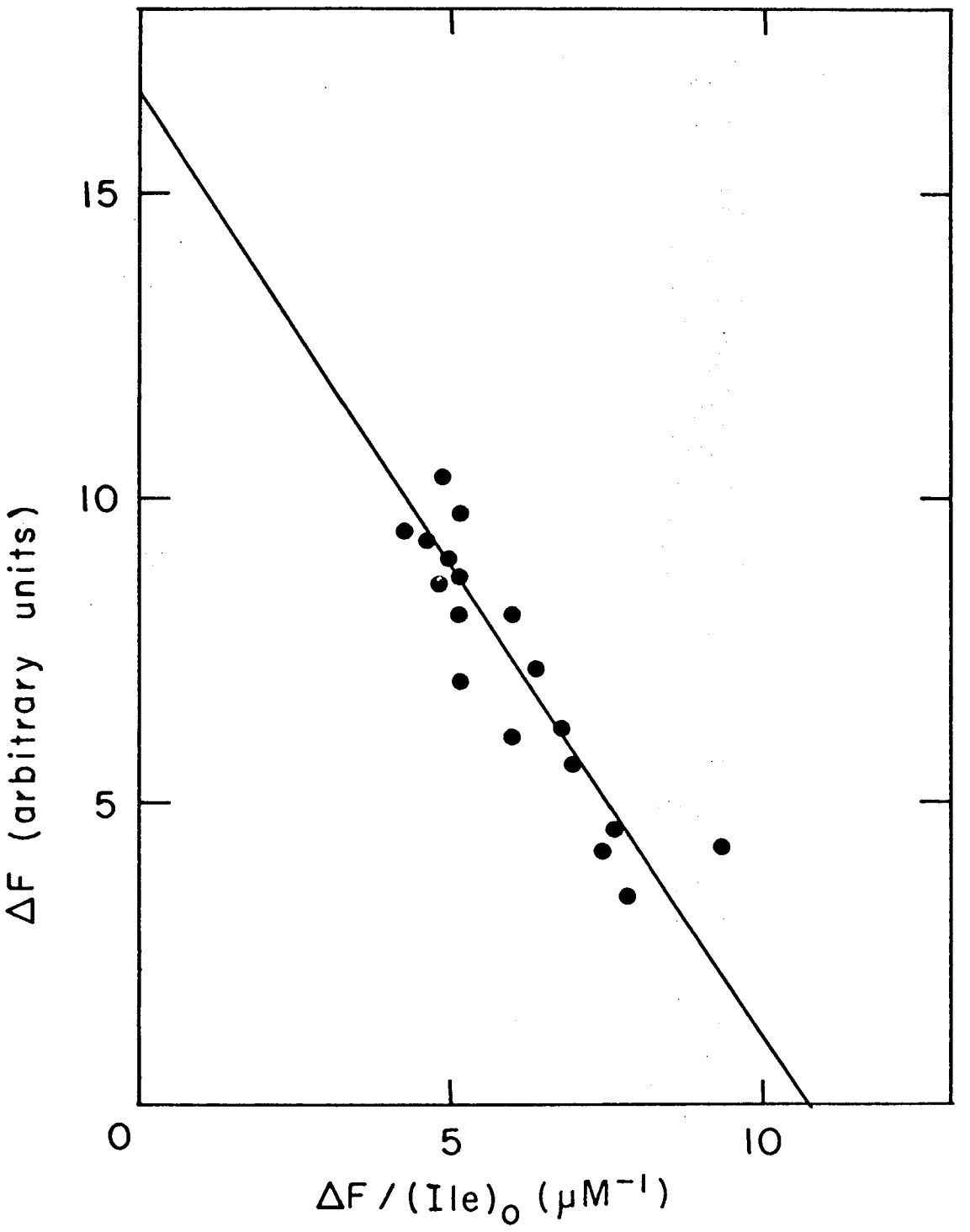


Fig. 7

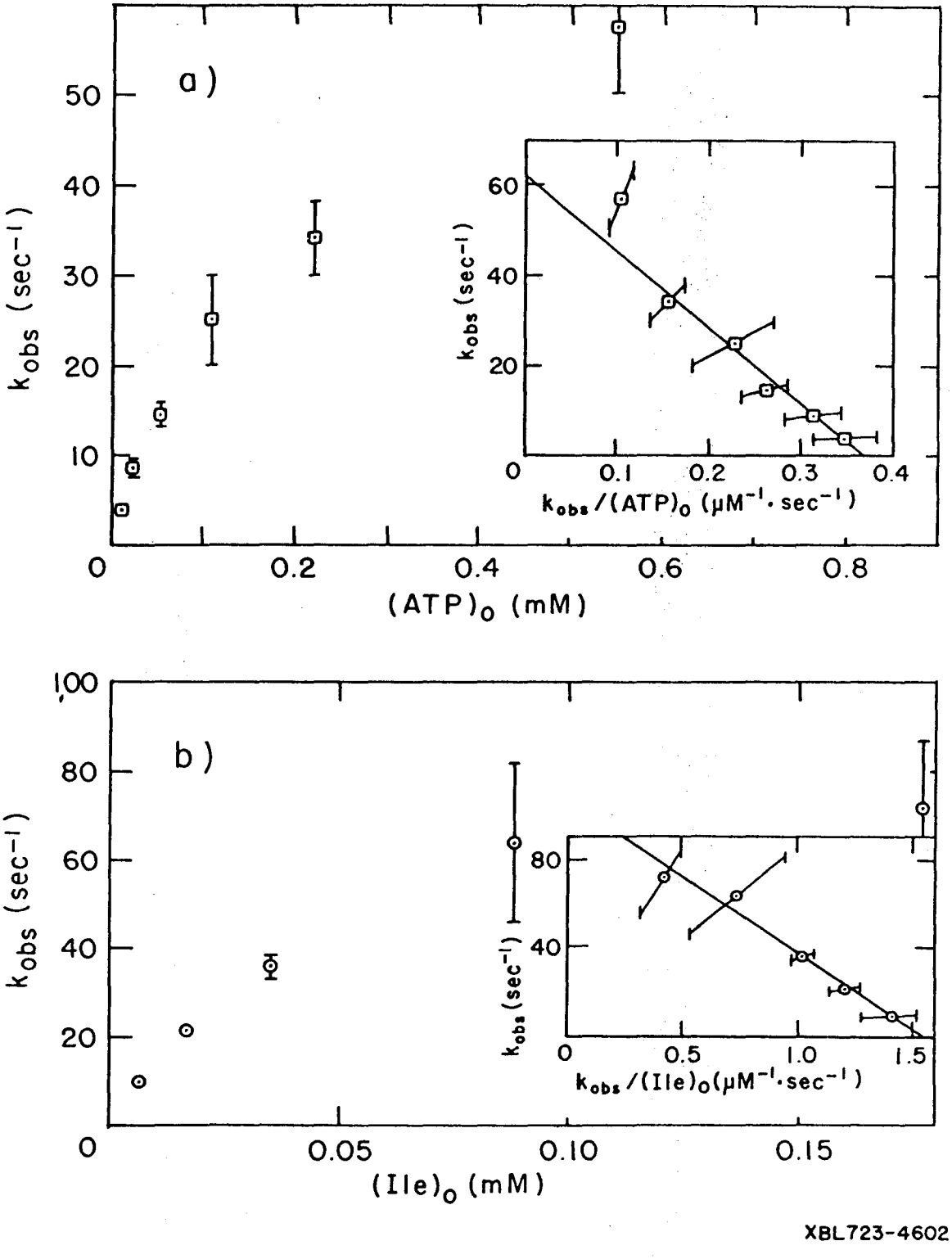

a)
k_{obs} (sec^{-1})
$(ATP)_o$ (mM)
k_{obs}/$(ATP)_o$ ($\mu M^{-1} \cdot sec^{-1}$)
b)
k_{obs} (sec^{-1})
$(Ile)_o$ (mM)
k_{obs}/$(Ile)_o$ ($\mu M^{-1} \cdot sec^{-1}$)
XBL723-4602

Fig. 8

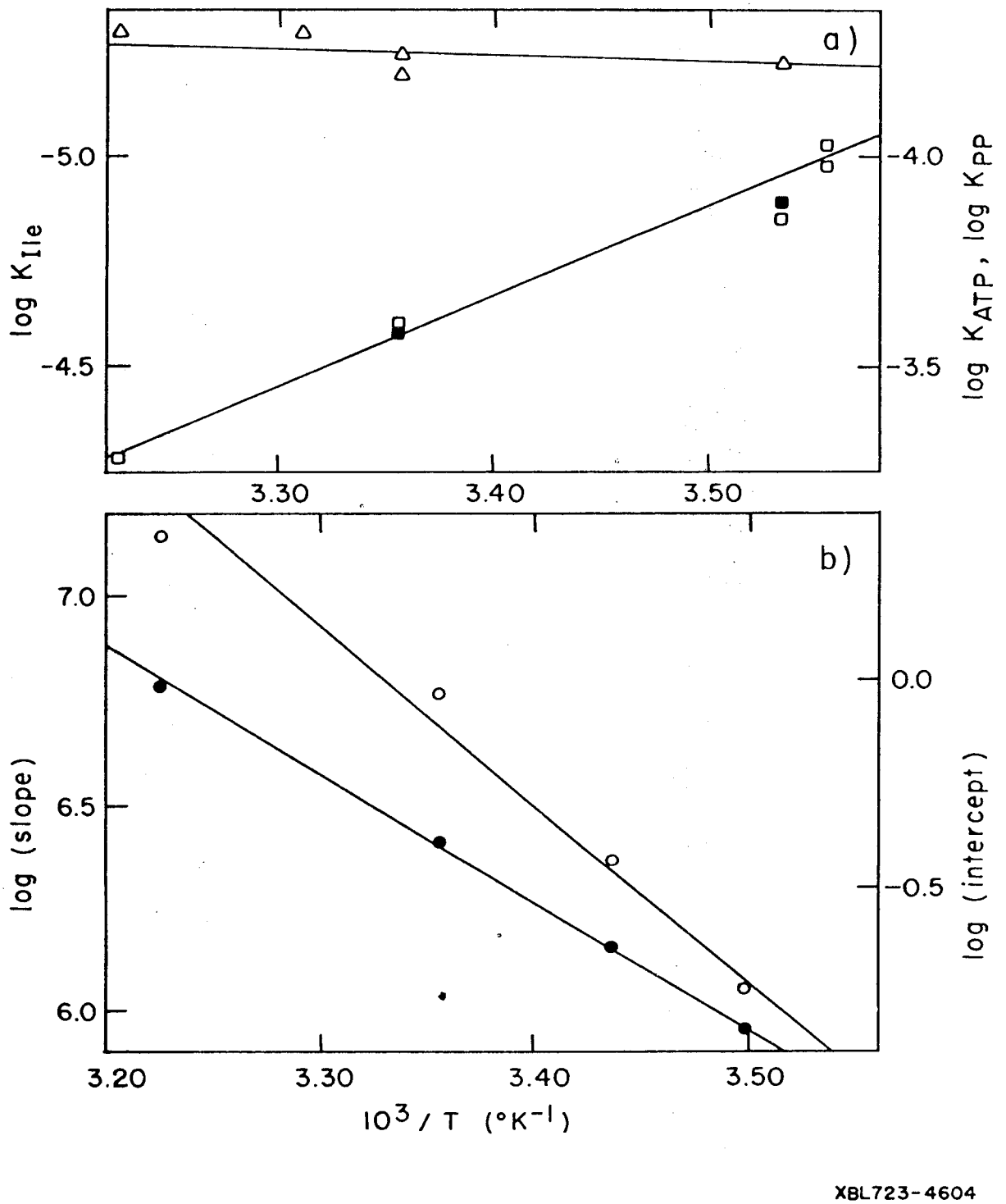

a)
-5.0
-4.5
log K_{Ile}
-4.0
-3.5
log K_{ATP}, log K_{PP}
3.30
3.40
3.50
b)
7.0
6.5
6.0
log (slope)
0.0
-0.5
log (intercept)
3.20
3.30
3.40
3.50
10^3 / T ($°K^{-1}$)
XBL723-4604

Fig. 9