

Kinetic analysis of the isoleucyl-tRNA synthetase mechanism: The next reaction cycle can start before the former ends.

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Running title:

Kinetic mechanism of isoleucyl-tRNA synthetase

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Enzyme: Isoleucyl-tRNA synthetase (EC 6.1.1.5)

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Abstract

A kinetic analysis of the isoleucyl-tRNA synthetase (IleRS) from *Escherichia coli* was accomplished. 16 different steady-state two-ligand experiments were statistically analysed simultaneously so that the same rate equations and same rate and dissociation constants applied to all experiments. The so called "rapid equilibrium segments" procedure was used in deriving the rate equations. The final best-fit mechanism included the normal activation and transfer steps, and reorganizing steps between them and after the transfer step. In addition the analysis strongly suggested an additional activation step, i.e. formation of a new isoleucyl-AMP before the isoleucyl-tRNA was freed from the enzyme. The removal of Ile-tRNA was possible without formation of Ile-AMP if both isoleucine and ATP were bound to the E·Ile-tRNA complex, but this route covered only 11 % of the total formation of Ile-tRNA. In addition to the Mg^{2+} ion in MgATP or MgPP_i, only two tRNA-bound Mg^{2+} ions were required to explain the magnesium dependence in the best-fit mechanism. The first of them could be present in all steps before the second activation and was obligatory in the first reorganizing step and transfer step. The second Mg^{2+} ion was present only at the transfer step whereas elsewhere it prevented the reaction, including the activation reactions. Chloride inhibited the IleRS reaction, 100 mM KCl caused 50 % inhibition if the ionic strength was kept constant with K-acetate. The K_m^{app} (tRNA) value was increased from 0.057 to 1.37 μ M when the KCl concentration was increased from 0 to 200 mM.

Introduction

The basic mechanism of the aminoacyl-tRNA synthetases includes the formation of aminoacyl-AMP in the activation reaction and thereafter the transfer of the amino acid from aa-AMP to tRNA in the transfer reaction. The basic mechanism was elucidated quite early after the discovery of the aminoacyl-tRNA synthetases [reviews 1,2]. Thereafter new details of the reaction have been the subject of continuous study, including the detailed crystal structure [3]. Much attention has been paid to the editing mechanisms which eliminate the erroneously formed products, pretransfer proofreading at the aa-AMP level and posttransfer proofreading at the aa-tRNA level [4,5]. The aminoacyl-tRNA synthetases have been divided in two classes according to structural and functional properties [6,7]. IleRS falls into Class Ia together with other synthetases for the branched chain amino acids.

A decade ago I published a best-fit analysis of the arginyl-tRNA synthetase reaction [8] where numerous two-ligand experiments were simultaneously analysed. In the present paper the same approach is repeated with the isoleucyl-tRNA synthetase from *Escherichia coli*. Both IleRS and ArgRS belong to the class I aminoacyl-tRNA synthetases and they have close structural similarities [9].

In some previous works I have applied the "rapid equilibrium segments" procedure to derive rate equations for aminoacyl-tRNA synthetases [10,11]. These analyses included the dependences on the magnesium and polyamine concentrations and the dissociation of Mg^{2+} ions from Ile-tRNA before it is freed from the enzyme [10], the differences in the magnesium dependences between the class I and class II aminoacyl-tRNA synthetases [11], and chloride inhibition [12]. Due to limited experimental material those studies did not lead to equations that could be satisfactorily used for new different experiments. In the present work the procedure has been improved by simultaneously analysing 16 different two-ligand experiments and, in addition, avoiding the inhibiting chloride and sulphate in adjusting the Mg^{2+} concentration. The optimal statistical result required a step at the end of the reaction cycle where Ile-AMP for the next cycle was formed before the Ile-tRNA was liberated from the enzyme. This revives the old reaction cycle suggested by Yarus and Berg [13]. Apparently the reaction site is opened when the CCA-Ile end of tRNA is turned to a separate editing site for the posttransfer proofreading [3]. So the formation of a new IleAMP could be possible on the reaction site before the Ile-tRNA is totally removed from the enzyme. This, also, increases the number of the enzyme intermediates which could be involved in the pretransfer proofreading.

Experimental procedures

Materials

Isoleucyl-tRNA synthetase was purified from *Escherichia coli* B as described previously [14]. Unfractionated tRNA from *E. coli* MRE 600 (Boehringer) was used.

Enzyme assays

The rate of the aminoacylation of tRNA was assayed with the filter paper - acid precipitation method [15] with modifications. The reaction mixture (100 μ l) contained 50 mM Hepes/25 mM KOH (pH 7.4 at 30 °C), 0.02 % chicken egg albumin, 1mg/ml of tRNA (1.1 μ M tRNA^{Ile}), 2 mM ATP, 5 μ M non-radioactive Ile, about 60 000 cpm of [¹⁴C]Ile (0.9 μ M), 3mM Mg-(acetate)₂ (1 mM excess Mg²⁺), 50 mM K-acetate, 1 mM dithiothreitol, and the enzyme. The reaction temperature was 30 °C. 180 μ M Mg²⁺ was carried to the reaction mixture by tRNA, and was taken into account in the calculations. The reaction was stopped by pipetting a sample (40 μ l) onto Whatman 3MM paper placed close to the surface of 50 % formic acid, which reduces the pH below 4 in seconds. The paper pieces were washed three times with a solution containing 0.2 M HCl and 5 % acetic acid, and finally once with ethanol. The paper pieces were counted for radioactivity of the formed aminoacyl-tRNA. The initial rates were calculated using the integrated Michaelis equation containing the product inhibition [16] to avoid the slight curvature in the rate curves caused by the formed Ile-tRNA.

The ATP - PP_i exchange activities were measured in a similar reaction mixture to the aminoacylation but ³²PP_i (50 000-300 000 cpm, 0.05 - 1 μ M) was substituted for the radioactive amino acid and 50 μ M non-radioactive PP_i was added. The Ile concentration was 50 μ M. The product radioactive ATP was separated from the radioactive PP_i by paper chromatography as described previously [14].

Equations and best-fit analysis

The equations were derived as described previously [8,10]. The following steps we included:

1. The total reaction scheme was divided in 4 - 6 segments, depending on the mechanism to be tested. The enzyme intermediates within a segment should be in equilibrium with each other. The *C* and *D* terms were defined to express the total enzyme intermediate concentrations in the segment ($D_i * [E_i]$) and the rates between the segments ($C_{ij} * [E_i]$).
2. The steady-state rate equations were derived for the ATP-PP_i exchange reaction and the aminoacylation of tRNA using the *C* and *D* terms. The segments were handled like the enzyme intermediates in the normal derivation of rate equations.

3. In the rate equation algorithm the C and D terms were expressed using the real rate and equilibrium constants and ligand concentrations.
4. The algorithm and the rate equations (from step 2) were used to obtain the calculated rate values. These calculated values and the measured rate values were used in the best-fit analysis.

The best-fit analysis was done using the non-linear regression with the least squares of the residuals between the measured and calculated rate values. The kinetic constants were refined by successive iterations. The variances and standard errors were calculated as described [8]. The sum of the variances of the different experiments was minimized. The minimization was done by systematically changing (in 5 %, 1 %, or 0.2 % steps) the value of a kinetic constant in the rate equation algorithm, choosing the value giving the lowest sum of variances, and doing the same with all (39) constants one by one. Since the values of the kinetic constants are not independent of each other, the procedure is repeated so many times that the sum of the variances and the values of the constants do not change. The order of the constants in the row should be varied, too.

Results

Measurements

The kinetic measurements for the statistical analysis are presented in Figs. 1 and 2. They all are two-ligand steady-state experiments where either the ATP - PP_i exchange or the aminoacylation of tRNA are measured. The experiments were chosen to cover the different parts of the total reaction. For instance the changes in the ATP and isoleucine concentrations (in segment 1) or PP_i concentrations (segment 2) or AMP concentrations (segment 3) describe mainly the events close to the segments where these ligands are bound. In the aminoacylation reactions (Fig. 2) the products PP_i and AMP inhibit, but in the ATP-PP_i exchange reaction PP_i works like substrate. The simultaneous analysis of all experiments requires that they have been done under similar conditions. The potassium concentration used here 75 mM, coming from 50 mM K-acetate and the buffer. Chloride and sulphate were avoided due to their inhibiting effect. Their concentrations are low in *E. coli* cells [17,18]. Mg-acetate was used instead of MgCl₂ or MgSO₄ to adjust the magnesium concentration. The substrate concentration dependences were plotted as Hanes plots (s/v vs. s) and the inhibition dependences as Dixon plots ($1/v$ vs. i). Due to the complexity of the reaction the Hanes and Dixon plots are seldom straight lines and the v vs. [Mg²⁺] plots are not hyperbolas.

In Fig. 3 the relation between the ATP - PP_i exchange and aminoacylation rates,

$v_{\text{exch}}/v_{\text{acyl}}$, was measured, and these results were used in the statistical analysis.

This relation gives a simple expression if the second activation reaction does not exist [8] but the second activation makes the relation more complicated.

Best-fit model

In the best-fit analysis the sum of the squares of the residuals between the measured and calculated rate values was used to calculate the error per cents for all two-ligand experiments in Figs 1 - 3. The sum of the error per cents was minimized.

The scheme of the best-fit model of the reaction is given in Fig. 4. The reaction is divided into six segments according to the procedure of the "rapid equilibrium segments" [19]. The reaction scheme follows mainly the known reaction mechanism [1-5] until segment 4, including the activation and transfer reactions and the binding modes of the substrates. The role of the separate posttransfer editing site, which as well is a known structure [3,5], comes evident in segments 5 - 6. It gives the possibility for the CCA-Ile end of tRNA and for IleAMP to be bound to different sites. Although there seems not to be any discrepancy between this structure and the present kinetic model, the kinetic details remain to be adjusted.

The total rate is slow ($k_{\text{cat}} = 0.7 \text{ s}^{-1}$) allowing the equilibrium to be settled in the different segments. The reaction can run either through segment 5 \rightarrow segment 1 or segment 5 \rightarrow segment 6 \rightarrow segment 2. The rate equation is an algorithm which comes from the scheme in Fig. 4. The rate of the aminoacylation of tRNA is expressed by Eq. 1. The rate of the ATP - PP_i exchange in the presence of tRNA is in Eq. 2 and in absence of tRNA in Eq. 3. The values of the kinetic constants in the best-fit system are in Table 1. If a ligand exists similarly in a segment and the reactions from and to the segment, the terms containing the ligand need not to be written in the equations. Therefore tRNA has not been written in D_3 and Ile-tRNA is not in D_4 , D_5 or D_6 .

If all rate constants are multiplied by the same number, the error per cents, and thus the whole best-fit system, remain the same, and the dissociation constants are not changed. The level of the rate constants was chosen to give the k_{cat} value measured at optimal conditions, 0.7 s^{-1} . In order to find the main route of the product dissociation the removal of the product Ile-tRNA from the enzyme complex is divided into seven different rate constants k_{8x} depending on the enzyme intermediate from which the product is removed.

Rate equation algorithm

In the algorithm ATP_f means ATP without Mg, and tRNA_f is tRNA without Mg.

$$\begin{aligned}
[\text{tRNA}_f] &= [\text{tRNA}_{\text{tot}}]/(1+([\text{Mg}^{2+}]/K_{\text{MR}})*(1+[\text{Mg}^{2+}]/K_{\text{MR}2})) \\
[\text{MgtRNA}] &= [\text{tRNA}_f]*([\text{Mg}^{2+}]/K_{\text{MR}}) \\
[\text{Mg}_2\text{tRNA}] &= [\text{tRNA}_f]*([\text{Mg}^{2+}]/K_{\text{MR}})*([\text{Mg}^{2+}]/K_{\text{MR}2}) \\
C_{12} &= k_3*(1+[\text{tRNA}_f]/K_{5F1}+[\text{MgtRNA}]/K_{51})*[\text{Ile}]/K_2*[\text{MgATP}]/K_{1M} \\
C_{21} &= k_{-3}*(1+[\text{tRNA}_f]/K_{5F2}+[\text{MgtRNA}]/K_{52})*([\text{MgPPi}]/K_{4M}) \\
C_{23} &= k_{6C}*[\text{MgtRNA}]/K_{52}*(1+[\text{MgPPi}]/K_{4M}) \\
C_{32} &= k_{-6C}*(1+[\text{MgPPi}]/K_{4M}) \\
C_{34} &= k_6*[\text{Mg}^{2+}]/K_{\text{ME}3} \\
C_{43} &= k_{-6} *[\text{AMP}]/K_{74}*([\text{Mg}^{2+}]/K_{\text{ME}4})*([\text{Mg}^{2+}]/K_{\text{ME}42}) \\
C_{45} &= k_{8C}*(1+[\text{Mg}^{2+}]/K_{\text{ME}4}) \\
C_{54} &= k_{-8C}*(1+[\text{Mg}^{2+}]/K_{\text{ME}5}) \\
C_{56} &= k_{35} *[\text{Ile}]/K_{25} *[\text{MgATP}]/K_{\text{AM}} \\
C_{65} &= k_{-35} *[\text{MgPPi}]/K_{4M6} \\
C_{51} &= k_8*(1+[\text{MgATP}]/K_{\text{AM}}+[\text{Ile}]/K_{25}) + k_{8SA} *([\text{Ile}]/K_{25}) *([\text{MgATP}]/K_{\text{AM}}) + \\
&k_{8M} *[\text{Mg}^{2+}]/K_{\text{ME}5} *(1+[\text{MgATP}]/K_{\text{AM}}) \\
C_{62} &= k_{86} + k_{86P} *[\text{MgPPi}]/K_{4M6} \\
D_1 &= ([\text{Mg}_2\text{tRNA}]/K_{5M1} + (1+[\text{tRNA}_f]/K_{5F1}+[\text{MgtRNA}]/K_{51})*(1+[\text{Ile}]/K_2)) *(1+[\text{MgATP}]/K_{1M} + \\
&[\text{ATP}_f]/K_1 + [\text{AMP}]/K_{71}) \\
D_2 &= (1+[\text{MgPPi}]/K_{4M}+[\text{PPi}]/K_4 +[\text{ATP}_f]/K_{A2})* (1+[\text{tRNA}_f]/K_{5F2}+[\text{MgtRNA}]/K_{52}) \\
D_3 &= (1 + [\text{Mg}^{2+}]/K_{\text{ME}3} + [\text{MgPPi}]/K_{4M}) \\
D_4 &= (1 + [\text{AMP}]/K_{74})* (1 + [\text{Mg}^{2+}]/K_{\text{ME}4})* (1 + [\text{Mg}^{2+}]/K_{\text{ME}42}) \\
D_5 &= (1 + [\text{Ile}]/K_{25} + [\text{Mg}^{2+}]/K_{\text{ME}5})* (1 + [\text{MgATP}]/K_{\text{AM}}) \\
D_6 &= (1+ [\text{MgPPi}]/K_{4M6}) \\
PAR5 &= C_{65}/C_{56}+C_{62}/C_{56} \\
PAR4 &= (C_{54}/C_{45}+C_{51}/C_{45})*PAR5+C_{62}/C_{45} \\
PAR3 &= C_{43}/C_{34}*PAR4+C_{51}/C_{34}*PAR5+C_{62}/C_{34} \\
PAR2 &= C_{32}/C_{23}*PAR3+C_{51}/C_{23}*PAR5+C_{62}/C_{23} \\
PAR1 &= C_{21}/C_{12}*PAR2+C_{51}/C_{12}*PAR5 \\
DENOM &= D_1*PAR1+D_2*PAR2+D_3*PAR3+D_4*PAR4+D_5*PAR5+D_6
\end{aligned}$$

$$v_{\text{acyl}} = (C_{51} * PAR5 + C_{62}) / DENOM \quad (1)$$

$$v_{\text{exch}} = (C_{21} * PAR2 + C_{65}) / DENOM \quad (2)$$

$$v_{\text{exch}} = C_{21}/(D_1 * C_{21}/C_{12} + D_2) \quad (3)$$

Description of the details of the reaction in different segments

Segment 1: The substrates isoleucine, MgATP and MgtRNA are written in D_1 to be bound in random order. Mg₂tRNA is bound but does not stay bound further in C_{12} , and thus inhibits. Free ATP and AMP compete with MgATP. The binding of the free tRNA (without Mg²⁺) is weak. The reaction C_{12} runs both in the presence and absence of MgtRNA.

Segment 2: In the best-fit system PP_i and tRNA can be bound simultaneously, or they do not compete. MgPP_i is the reacting form. Mg₂PP_i is not bound, its dissociation constant becomes high in the statistical analysis. Mg₂tRNA is not bound, either. In D_2 the free ATP is written to compete with PP_i. It improves the fit, especially in the ATP - PP_i exchange experiments. MgATP does not have the same effect. The binding of ATP to the PP_i site exists in the formation of diadenosine tetraphosphate (Ap₄A) [20] but this is not further tested here. In the next C_{23} step MgtRNA must be present. MgPP_i is not necessary but does not inhibit the step.

Segment 3: One Mg²⁺ ion is coming to segment 3 in MgtRNA. Another Mg²⁺ ion is bound to the complex at this segment. The next step, the transfer reaction, requires two bound Mg²⁺ ions. MgPP_i is not necessary in this segment but it does not inhibit either. In the next C_{34} reaction MgPP_i is not present.

Segment 4: AMP and one Mg²⁺ ion must dissociate from the E-IletRNA complex in segment 4. The other Mg²⁺ ion can also dissociate, but can as well be present in the further C_{45} reaction.

Segment 5: The best-fit mechanism requires strong binding of Mg²⁺ in segment 5. This can be the same Mg²⁺ ion as before in C_{45} but the binding is about 13 times stronger. The Mg²⁺ ion in segment 5 competes with isoleucine (not necessarily for the same binding site), and the presence of Mg²⁺ almost totally prevents the further reactions C_{51} and C_{56} . The further reactions require that both isoleucine and ATP are bound. That is, naturally, obligatory on the route segment 5 → segment 6 → segment 2, but even on the route segment 5 → segment 1 as much as 93 % of the reaction goes through the intermediate IletRNA-E(Ile)(ATP).

Step C_{56} is another activation reaction (like step C_{12}) for the next reaction cycle. The position of the posttransfer proofreading should be at segment 5, and therefore D_5 in fact is more complicated. Apparently the synthetic site is opened for new Ile and ATP when the -CCA-Ile end of the tRNA turns to the editing site. The removal of Mg²⁺ (K_{ME5}) may have a role in the opening.

Segment 6: Both Ile-tRNA and Ile-AMP stay attached to the enzyme. The best-fit analysis suggests a random dissociation order of PP_i and Ile-tRNA from the enzyme at step C₆₂.

The calculated amounts of the enzyme intermediates in different segments show that 84 % of the enzyme is in segment 2 and 6.6 % in segment 6 in the absence of PP_i and AMP.

Chloride inhibition

Chloride inhibits the IleRS reaction (Fig. 5). The inhibition is prominent at about a 1 mM concentration of free Mg²⁺ (i.e. 3 mM in Fig. 5A, Mg²⁺_{free} + MgATP = 3 mM), which is the concentration of free Mg²⁺ in the cell [21,22,23]. High Mg²⁺_{free} concentrations (>10 mM) almost eliminate the chloride inhibition. K-acetate inhibits weakly but K₂SO₄ stronger than KCl (Fig. 5B). KCl and K-acetate inhibit above 50 mM concentrations when high enough K⁺ concentration for full activity has been reached. If the K⁺ concentration is kept constant at 200 mM the inhibitory effect of KCl starts from low concentrations. 50 mM K-acetate was used throughout the present work to keep the K⁺ amount high enough (75 mM together with the buffer).

KCl affects the K_m (tRNA) value. The K_m values were 0.057, 0.129, 0.334, 0.657, and 1.37 μ M at KCl concentrations of 0, 50, 100, 150, and 200 mM, respectively.

The best-fit analysis for chloride effects was done using three types of experiments: the K_m (tRNA) assay, and the experiments similar to Fig. 2H and Fig. 2I at 50 mM and 150 mM KCl. 150 mM KCl caused an approximately eight times increase in the value of K_{MR2} and a twofold increase in K_{ME42} . The value of K_{S2} was 0.3, 0.5 or 2.0 μ M at 0, 50, and 150 mM KCl, respectively.

The chloride inhibition in IleRS is somewhat stronger than in the arginyl-tRNA synthetase [8] and tyrosyl-tRNA synthetase [12]. Essentially similar chloride inhibition was observed in the class II synthetases for phenylalanine, serine, histidine and lysine (not shown) and seems to be a general feature of the aminoacyl-tRNA synthetases, at least in *E. coli*. The chloride concentration in growing *E. coli* is low (below 25 mM [18] or "virtually absent" [17]). Chloride inhibition might make a connection between the rate of protein synthesis and the function of chloride channels and cell potentials [24,25].

Effect of spermidine

Polyamines can replace part of the Mg²⁺ ions in the aminoacyl-tRNA synthetase reactions [26,27,11]. Polyamines were not included in the above experiments for the statistical analysis. Only one experiment with spermidine is presented here (Fig. 6). Its analysis was carried out using the above mechanism and the constant values from Table 1, but spermidine was set to compete with the Mg²⁺ ions in the equations. Only two competition sites were important. Spermidine could replace

Mg^{2+} (K_{ME3}) and Mg^{2+} (K_{ME42}) which are involved in the forward and backward transfer reaction. The corresponding dissociation constant values were $K_{SE3} = 470 \mu M$ and $K_{SE42} = 390 \mu M$. The competition with Mg^{2+} (K_{MR}) was much weaker, $K_{SR} = 2800 \mu M$. At the other binding sites no competition was observed.

Other reaction mechanisms

The above reaction mechanism is the best found so far. If the sum of the error per cents is used as a figure of merit (FOM), it gives the lowest reading, 83.9 per cent units. Some changes can be made in segment 5 without essential weakening the FOM value. If isoleucine and Mg^{2+} (K_{ME5}) do not compete, the FOM is 86.6. If $[Mg^{2+}]/K_{ME5}$ is removed from C_{54} , the FOM is 86.8. In that case Mg^{2+} (K_{ME5}) is not on the same binding site as Mg^{2+} (K_{ME42}), and the backward rate constant k_{-8C} becomes higher, about as high as k_{+8C} . When Mg^{2+} (K_{ME5}) is removed from segment 5, the FOM rises to 108.

When segment 6 is removed, or the reaction runs through segment 5 \rightarrow segment 1, the FOM is 90, but even then 99 % of the reaction runs through the Ile-tRNA-E(ATP)(Ile) complex where both ATP and Ile are bound. If ATP is then removed from segment 5, the FOM is 228 and if Ile is removed it is 128, and if Mg^{2+} (K_{ME5}) is removed, 145.

In all the above cases the optimization of all constants was carried out, not only the constants involved in the mentioned change.

Discussion

Reaction cycle with formation of second aaAMP before dissociation of the product aa-tRNA

In one of the oldest kinetic works on IleRS Yarus and Berg [13] studied the binding of tRNA, Ile-tRNA and modified tRNA:s to the enzyme, and were led to suggest a sequence where the Ile-AMP for the next reaction cycle was formed before the Ile-tRNA was freed from the enzyme complex. Eldred and Schimmel [28] also deduced the same reaction cycle. In a thorough kinetic analysis by Freist et al. [29] a similar model was one of the possible reaction cycles. Generally, however, such an early formation of Ile-AMP has not been included in the proposed reaction mechanisms.

An analogous case was found with ArgRS which normally requires the presence of tRNA for the ATP - PP_i exchange, but the exchange reaction was continued after cessation of tRNA, in the presence of Arg-tRNA [8].

Although IleRS is an one-subunit enzyme and has one reaction site, it has an additional editing site for elimination of wrong amino acids [3,5]. The CCA-Ile end of the Ile-tRNA is turned from the reaction site to the editing site. It looks like the reaction site could be opened for a new Ile-AMP when the CCA-Ile end is on the editing site.

The present work suggests that Ile-tRNA is freed from the enzyme either without formation of Ile-AMP in reaction C_{51} , or after its formation in C_{62} . The calculated rates show that 89 % of the Ile-tRNA comes through the route segment 5 \rightarrow segment 6 \rightarrow segment 2 and only 11 % through segment 5 \rightarrow segment 1 (at 2 mM MgATP, 50 μ M Ile and 1 mM free Mg^{2+}). Moderate changes in the ligand concentrations do not much change this relation. Only PP_i lowers the relation, at 50 μ M PP_i it is 80 %.

Number of participating Mg^{2+} ions

ATP and PP_i always exist in the aminoacyl-tRNA synthetase reactions as MgATP and $MgPP_i$, but Mg_2PP_i seems not to have importance in the IleRS reaction.

Generally, several Mg^{2+} ions are bound to tRNA [30-33] and are involved in its correct folding [34-36]. However only few of the tRNA-bound Mg^{2+} ions are kinetically important [11]. Only two tRNA-bound Mg^{2+} ions are included in the present analysis of IleRS, and one or two Mg^{2+} ions are attached at the same time to the E-tRNA complex in different steps of the reaction. The equations contain six Mg^{2+} ions (in addition of those in MgATP or $MgPP_i$), but some of them must have common binding sites. The terms $[Mg^{2+}]/K_{ME3}$ and $[Mg^{2+}]/K_{ME42}$ must refer to the same binding site since both participate only in the transfer reaction, one forward and the other backward, and in addition, spermidine can replace both of these Mg^{2+} ions. This binding not only promotes the transfer reaction but prevents the other steps, including the activation reactions. According to the equation, the ion Mg^{2+} (K_{MR2}) could have the same site, too, although it represents the binding to tRNA and not to the E-tRNA complex. Chloride weakens the binding of Mg^{2+} (K_{MR2}) and Mg^{2+} (K_{ME42}).

Mg^{2+} (K_{MR}) and Mg^{2+} (K_{ME4}) form another pair which probably bind to a common site. They cannot be replaced by spermidine, and chloride does not weaken their binding. In the ordered binding to the free tRNA, Mg^{2+} (K_{MR}) is bound first, and in the ordered dissociation in segment 4 Mg^{2+} (K_{ME4}), it is freed last.

It is an open question whether Mg^{2+} (K_{ME4}) and Mg^{2+} (K_{ME5}) have the same binding site. According to the equations that could be possible, even though the latter is bound 13 times more

strongly. The other possibility, that of having different sites, can also be correct, since removing the term $[Mg]/K_{ME5}$ from C_{54} leads to only a slightly weaker result in the best-fit analysis.

In conclusion, in addition to the Mg^{2+} ion in $MgATP$ or $MgPP_i$, two or three Mg^{2+} ions participate in the IleRS reaction. One of them can be present in all steps but must be present in the transfer reaction and the reorganizing step before it. The second of them is present only in the transfer reaction and prevents for example, the activation reaction. A strongly bound Mg^{2+} ion is involved in the events after the transfer reaction.

Relation to the pretransfer proofreading

During the whole time span of studies on IleRS understanding the discrimination of the non-cognate valine has been one of the key objectives [4]. The correction mechanisms include the pretransfer proofreading, where the wrong Val-AMP is eliminated, and posttransfer proofreading, where the wrong Val-tRNA^{Ile} is eliminated. The posttransfer proofreading has been shown to occur at a separate editing site where the noncognate aminoacids are hydrolysed [3].

In recent years the pretransfer proofreading has received new attention [37-39]. Although it occurs at the aa-AMP level, it requires tRNA and is believed to be connected to the posttransfer proofreading site. In the present work measurements of the proofreading were not done. However, the existence of both Ile-AMP and Ile-tRNA in a ternary complex expands the number of enzyme intermediates which could be involved. If the pretransfer proofreading occurs at segment 6, before the dissociation of Ile-tRNA from the enzyme, the connection to the posttransfer editing site could be possible through Ile-tRNA.

The kinetic proofreading system has been a subject of discussion and mathematical calculations [40-42]. It requires either an increased pyrophosphorolysis rate of the wrong aa-AMP (in C_{65} and C_{21}) or dissociation of the wrong aa-AMP from the enzyme and hydrolysis thereafter. These rates can be different in the presence of Ile-tRNA in segment 6 from that in segment 2. In addition, the $Mg^{2+}(K_{ME5})$ ion in the presence of Ile-tRNA can affect the rates.

Still another possibility for the pretransfer proofreading is a hydrolytic elimination of the wrong aa-AMP on the synthetic site. Even then, the presence of Ile-tRNA and the Mg^{2+} ion can have their effects.

In the cell a major part of tRNA is as aa-tRNA [43]. A rough estimate of the strength of binding of Ile-tRNA to the enzyme can be calculated by analysing the rate curve of the aminoacylation assay by the integrated Michaelis equation [16]. This gives the result that the K_m for tRNA is about twice the value of K_p for Ile-tRNA, or Ile-tRNA is bound more strongly to the enzyme. The high amount of Ile-tRNA and its stronger binding mean that the amounts of enzyme intermediates with

bound Ile-tRNA in segments 6 and 5 get higher compared to segment 2. This emphasizes segment 6 as the possible site of the pretransfer proofreading.

For the kinetic proofreading some PP_i must be present in C_{65} and C_{21} . Due to the fast pyrophosphorolysis rates (high k_{-3} and k_{-35}) the required PP_i amount is low, estimated to be below 20 μ M. High PP_i concentrations (0.5 mM) have been repeatedly been measured in *E. coli* cells [44]. They cause such strong inhibition that, apparently, high PP_i concentrations cannot be in contact with the enzyme. PP_i increases the pyrophosphorolysis rates in C_{21} and C_{65} towards free Ile, and therefore the advantage of pretransfer proofreading is lost when the corrected Ile from IleAMP is mixed with the original mixture of amino acids.

Concluding remarks

The key aim of the present work has been to do a best-fit analysis simultaneously on a large number of different steady-state kinetic experiments and thus improve the model of the reaction mechanism and the accuracy of the equations. Several improvements could be done, although this kind of kinetic work, naturally, cannot address the mechanism at the molecular level but only at the functional level.

The procedure of the "rapid equilibrium segments" was used in the work. It can be seen that the equations in the procedure rapidly become complicated when the number of the segments increases. The rate equation algorithm contained 25 separate equations. On the other hand, the aminoacyl-tRNA synthetases are among the most complicated enzymes with three substrates, three products, activators and editing mechanisms, nonetheless IleRS could be successfully analysed. The principal advantage of the procedure is that only a limited number of equations in the algorithm must be modified when the details of the mechanism in a given segment are tested.

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Table 1

The best-fit values of the kinetic constants of the IleRS reaction.

The k_{cat} value at optimal reaction conditions was 0.71 s^{-1} .

Constant	Definition	unit	value	range
K_{1M}	MgATP·E	μM	371	300 - 440
K_1	free ATP·E	μM	1043	500 -
K_{AM}	MgATP·E(IletRNA)	μM	2077	1820 - 2400
K_{A2}	free ATP·E(IleAMP)	μM	746	610 - 940
K_2	Ile·E	μM	6.63	5.8 - 7.3
K_{25}	Ile·E seg. 5	μM	9.28	8.1 - 10.5
k_{+3}	activation	s^{-1}	37.7	35.1 - 41.1
k_{-3}	pyrophosphorolysis	s^{-1}	326	313 - 338
k_{+35}	activation, seg. 5	s^{-1}	62.8	54 - 72
k_{-35}	rev. activation, seg. 5	s^{-1}	326	210 - 420
K_{4M}	MgPP·E	μM	339	327 - 353
K_4	free PP_i ·E	μM	46.6	29 - 97
K_{4M6}	MgPP·E(IletRNA)	μM	258	< 930
K_{5M1}	Mg ₂ tRNA·E, seg.1	μM	0.075	0.065 - 0.091
K_{51}	MgtRNA·E, seg. 1	μM	0.206	0.17 - 0.25
K_{5F1}	free tRNA·E, seg.1	μM	1.7	
K_{52}	MgtRNA·E, seg.2	μM	0.297	0.282 - 0.312
K_{5F2}	free tRNA·E, seg.2	μM	4.7	
k_{+6C}	conf. ch.	s^{-1}	6.81	6.55 - 7.09
k_{-6C}	conf. ch.	s^{-1}	81.1	77 - 85
k_{+6}	transfer	s^{-1}	32.2	30.6 - 33.8
k_{-6}	reverse transfer	s^{-1}	299	272 - 327
K_{71}	AMP·E, seg.1	μM	705	360 <
K_{74}	AMP·E seg. 4	μM	2198	2020 - 2410
k_{+8C}	conf. ch.	s^{-1}	8.39	7.7 - 9.3
k_{-8C}	conf. ch.	s^{-1}	0.436	0.34 - 0.54

k_{+8}	IletRNA·E, seg. 5	s^{-1}	0.048	< 0.29
k_{+8ATP}	IletRNA·E(ATP), seg.5	s^{-1}	0.02	< 4.1
k_{+8ILE}	IletRNA·E(Ile), seg.5	s^{-1}	0.466	0.11- 0.82
k_{+8M}	IletRNA·E(Mg), seg. 5	s^{-1}	0.022	0.014 - 0.03
k_{+8SA}	IletRNA·E(Ile)(ATP), seg.5	s^{-1}	7.0	< 15.7
k_{+86}	IletRNA·E(IleAMP), seg.6	s^{-1}	4.15	2.5 - 10.3
k_{+86P}	IletRNA·E(IleAMP)(MgPP)	s^{-1}	262	170 - 440
K_{MR}	Mg·tRNA	μM	2489	2160 - 2820
K_{MR2}	MgtRNA·Mg	μM	1635	1480 - 1830
K_{ME3}	Mg·E(MgtRNA)(IleAMP)	μM	2580	2460 - 2710
K_{ME4}	Mg·E(IletRNA)	μM	1515	1200 - 1890
K_{ME42}	Mg·E(IletRNA)(Mg)	μM	6095	5570 - 6720
K_{ME5}	Mg·E(IletRNA)	μM	117	103 - 132

Fig. 1. ATP - PP_i exchange rates at different conditions. The lines were calculated using the rate equation algorithm, equations (2) or (3), and the optimized constant values from Table 1. The total Mg²⁺ ion concentrations were in A, [MgATP]+1 mM; C, [MgATP]+[MgPP_i]+1 mM; D, [MgPP_i]+[Mg-acetate]; E, [MgPP_i]+4 mM; and in B and F as shown. tRNA was not present in A, B, C and D. The standard errors were A, 2.1%; B, 3.2%; C, 4.2%; D, 4.7 %; E, 3.0% and F, 7.4 %.

Fig. 2. Aminoacylation of tRNA at different conditions. Equation (1) was used in the calculations of the lines. The total Mg²⁺ ion concentrations were in A, [MgATP]+4 mM; B as shown; C, [MgPP_i]+4 mM; D, [MgATP]+8 mM; E, [MgATP]+2 mM; F, [MgATP]+1 mM; G, 4 mM; H, [Mg-acetate]+1 mM; I, [MgATP]+[Mg-acetate]. The standard errors were in A, 5.0%; B, 9.8%; C, 5.3%; D, 6.7%; E, 5.1%; F, 3.7%; G, 3.7%; H, 6.9%; and I, 6.1%.

Fig. 3. Relation of the rates of ATP - PP_i exchange and aminoacylation of tRNA. The lines were calculated using equations (1) and (2) and the optimized constant values. The exchange and aminoacylation rates were measured at identical conditions, only the radioisotope was changed. ATP was 2 mM, Ile 10 μM, and total Mg²⁺ was 4 mM + [MgPP_i]. The standard error was 7.0 %.

Fig. 4. Division of the isoleucyl-tRNA synthetase reaction in six segments. The central enzyme intermediate and the ligands which are bound to it in each segment are indicated. The sum of the enzyme intermediate concentration in the segment *i* is $D_i \cdot [E_i]$ where E_i is the central intermediate. The rate from segment *i* to segment *j* is $C_{ij} \cdot [E_i]$. The expressions of the D_i and C_{ij} terms are written in the rate equation algorithm.

Fig. 5. Chloride effect on the aminoacylation reaction. In A 50 mM K-acetate was present in the reaction mixture but in B only when indicated. In the dashed line the KCl concentration is indicated and K⁺ ion concentration is kept at 200 mM with K-acetate. ATP was 2 mM, Ile 5 μM and in B Mg-acetate was 3 mM.

Fig. 6. Spermidine effect on the aminoacylation of tRNA. The curves were plotted using the values of the constants from Table 1 and setting [Spd]/ K_{SE3} to compete with [Mg²⁺]/ K_{ME3} and [Spd]/ K_{SE42} to compete with [Mg²⁺]/ K_{ME42} .