Editing Mechanisms in Protein Synthesis. Rejection of Valine by the Isoleucyl-tRNA Synthetase†

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ABSTRACT: Although the isoleucyl-tRNA synthetase from *Escherichia coli* (IRS) does not catalyze the overall mischarging of $tRNA^{Ile}$ with valine, it does undergo the first step of the reaction, the formation of an IRS·Val-AMP complex. The addition of $tRNA^{Ile}$ to this complex leads to its quantitative hydrolysis and the IRS acts as an ATP pyrophosphate in the presence of valine and $tRNA^{Ile}$ (Baldwin, A. N., and Berg, P. (1966), *J. Biol. Chem. 241,* 839). It is shown that during the ATP pyrophosphatase reaction: (a) IRS forms an IRS·Val-AMP complex; (b) the turnover number of the ATP pyrophosphatase reaction is the same as the rate constant for the transfer of isoleucine from IRS·Ile-AMP to $tRNA^{Ile}$ over a wide range of temperature and pH; (c) mischarged Val-$tRNA^{Ile}$ is hydrolyzed by IRS with a turnover number of 10 s^{-1} at pH 7.78 and 25 °C, compared with a value of 1.2 s^{-1} for the transfer of isoleucine from IRS·Ile-AMP to tRNA or for the ATP pyrophosphatase reaction. Although this appears to be consistent with an editing mechanism in which there is a slow transfer of the valine from the IRS·Val-AMP to $tRNA^{Ile}$ followed by the rapid hydrolytic step, as recently found for the rejection of threonine by the valyl-tRNA synthetase, there is an inconsistency. This scheme predicts that on mixing IRS·[^{14}C]Val-AMP with $tRNA^{Ile}$ there should be a transient misacylation of the tRNA such that about 10% of the [^{14}C]Val is present as [^{14}C]Val-$tRNA^{Ile}$ at the peak. But 0.8% or less is found. This could possibly be caused by the IRS having a higher hydrolytic activity during the mischarging reaction than is measured on mixing the unligated enzyme with preformed Val-$tRNA^{Ile}$. Alternatively, a two-stage editing mechanism must be considered in which the majority of the Val-AMP is destroyed *before* the transfer to tRNA in the major editing step, while the hydrolytic activity of the IRS towards Val-$tRNA^{Ile}$ is a second editing step to mop up any mischarged tRNA formed by the Val-AMP escaping the first editing step. It is shown that the "kinetic proofreading" mechanism of Hopfield is not consistent with the experimental data.

During protein synthesis the cell prevents the net misacylation of $tRNA^{Ile}$ with valine, and $tRNA^{Val}$ with threonine by using editing mechanisms to hydrolyze the undesired products or intermediates (Baldwin and Berg, 1966; Fersht and Kaethner, 1976b). This complex procedure is necessary because there are insufficient structural differences between the pairs of amino acids to discriminate between them by a simple mechanism. The rejection of threonine by the valyl-tRNA synthetase (VRS[1]) from *Bacillus stearothermophilus* has been shown to be due to the transient misacylation of the tRNA followed by its rapid hydrolysis (Fersht and Kaethner, 1976b).

The present study concerns the isoleucyl-tRNA synthetase (IRS) from *Escherichia coli* K12. This does not catalyze the aminoacylation of $tRNA^{Ile}$ with valine in experiments in vitro, and the overall error rate in vivo for the misincorporation of valine into the positions occupied by isoleucine in proteins is about 3 in 1×10^4 (Loftfield and Vanderjagt, 1972). The IRS forms a stable enzyme-bound valyl adenylate, which may be isolated by gel filtration. But whereas the addition of $tRNA^{Ile}$ to the IRS·Ile-AMP complex leads to the transfer of the isoleucine to the tRNA, the addition to IRS·Val-AMP leads to its net hydrolysis (Baldwin and Berg, 1966). In the presence of $tRNA^{Ile}$, ATP, and valine, the IRS acts an an ATP pyrophosphatase, hydrolyzing ATP to AMP (Baldwin and Berg, 1966). In the present study, the mechanism of the hydrolytic reaction is investigated using the ATP pyrophosphatase activity and rapid quenching and sampling experiments. As a prelude to this, we have shown that the mechanism of the formation of Ile-$tRNA^{Ile}$ involves the rapid formation of the IRS·Ile-AMP complex followed by the rate-determining transfer of the isoleucine to the tRNA (Fersht and Kaethner, 1976a).

Experimental Procedures

Materials and apparatus have been described previously (Fersht and Kaethner, 1976a). Unfractionated tRNA which had an isoleucine acceptance of 50 pmol/A_{260} was used, unless otherwise stated. Partially purified tRNA was obtained from M.R.E. Porton Down, Wilts, England. This had been enriched for $tRNA^{Ile}$ by fractionation on BD-cellulose in 10 mM $MgCl_2$ and 1 mM sodium thiosulfate with a gradient of 0.35–1.1 M NaCl, and 1.1 M NaCl–2.0 M NaCl, 20% ethanol. The $tRNA^{Ile}$ eluted at about 0.67 M NaCl with a second fraction at 1.33 M NaCl, 5% ethanol. The second fraction (isoleucine acceptance = 90 pmol/A_{260}) was further purified by chromatography on Sepharose 4B (Holmes et al., 1975; eluting at about 0.9 M $(NH_4)_2SO_4$) and RPC-5 (Kelmers and Heatherly, 1971; eluting at about 0.58 M NaCl) to an isoleucine acceptance of 1100 pmol/A_{260}.

Preparation of [^{14}C]*Val-tRNA^{Ile}*. (a) Using Fractionated $tRNA^{Ile}$ (cf. Giegé et al., 1974). A solution (4.8 mL) containing 1.6 A_{260} units of purified $tRNA^{Ile}$, valyl-tRNA synthetase from *Bacillus stearothermophilus* (0.21 mg), $MgCl_2$ (4.2 mM), ATP (0.83 mM), [^{14}C]Val (19 μM, 280 mCi/mmol), Tris-Cl (60 mM, pH 7.78), mercaptoethanol (10 mM), phenylmethanesulfonyl fluoride (0.1 mM) and dimethyl sulfoxide (33%, v/v) was incubated at 52 °C for 5 min. After cooling to 0 °C and adding 200 μL sodium acetate buffer (pH

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[1] Abbreviations used are: VRS and IRS, valyl- and isoleucyl-tRNA synthetases; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Bistris, 2-[bis(2-hydroxethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; ATP and AMP, adenosine tri- and monophosphates.

5.0, 1 M) tRNA was extracted by addition of a saturated solution of phenol (5 mL). After removal of the phenol by precipitation with 2 volumes of ethanol followed by gel filtration on Sephadex G-25 equilibrated with 10 mM $MgCl_2$, the resultant tRNA was found to be acylated with 1020 pmol of [^{14}C]Val per A_{260} unit.

(b) Using Unfractionated tRNA. The amino acid acceptance of all the tRNAs other than $tRNA^{Ile}$ was destroyed as follows. Unfractionated tRNA (isoleucine acceptance = 30 pmol/A_{260}) was deacylated by incubating for 1 h at 45 °C and pH 8.8 (50 mM Tris-Cl, 10 mM $MgCl_2$). The $tRNA^{Ile}$ was aminoacylated by incubating 2400 A_{260} units of tRNA for 3 min at 25 °C in a solution (12 mL) containing IRS (3 mg), isoleucine (100 μM), ATP (1 mM), Tris-Cl (pH 7.78, 144 mM), $MgCl_2$ (10 mM), mercaptoethanol (10 mM), and phenylmethanesulfonyl fluoride (0.1 mM). After the addition of sodium acetate (pH 5.0), phenol extraction, ethanol precipitation, and washing with 75% ethanol, the tRNA species other than the Ile-$tRNA^{Ile}$ were periodate oxidized by dissolving the tRNA in 30 mL of sodium acetate buffer (pH 5.0, 100 mM), adding $NaIO_4$ (21 mg) and incubating for 30 min in the dark. After the addition of 100 μL of glycerol and incubating for a further 5 min, the tRNA was precipitated with ethanol and washed. The oxidized terminal adenosine moieties and the isoleucyl residues of Ile-$tRNA^{Ile}$ were removed by incubation for 2 h at 45 °C in 0.5 M lysine (pH 9.0). After this treatment, the isoleucine acceptance was found to be 26 pmol/A_{260}, while the valine acceptance had dropped from 90 to less than 0.3 pmol/A_{260}. The $tRNA^{Ile}$ was misacylated with [^{14}C]Val by incubating a solution (4.5 mL) containing tRNA (70 A_{260} units), valyl-tRNA synthetase from *B. stearothermophilus* (1 mg), $MgCl_2$ (4 mM), ATP (1.1 mM), [^{14}C]Val (20 μM, 280 mCi/mmol), Tris-Cl (55 mM, pH 7.78), inorganic pyrophosphatase (10 units), mercaptoethanol (10 mM), phenylmethanesulfonyl fluoride (0.1 mM), and dimethyl sulfoxide (25%, v/v) for 10 min at 52 °C. After isolating the tRNA in 10 mM $MgCl_2$ as described previously, the material was found to be misacylated with 16 pmol of [^{14}C]Val/A_{260}.

[^{14}C]Val-$tRNA^{Ile}$ was found to be stable on storage at −20 °C.

Kinetic Methods. Unless otherwise stated, all buffers contained 10 mM $MgCl_2$, 0.1 mM phenylmethanesulfonyl fluoride, and 10 mM mercaptoethanol. The standard buffers were: pH 7.78, Tris-Cl (144 mM); pH 6.8, sodium cacodylate (100 mM); pH 5.87, Bistris-Cl (13 mM).

Preparation of Enzyme-Bound Aminoacyl Adenylates. Stock solutions of enzyme–aminoacyl adenylate complexes were prepared daily and stored on ice. A solution (0.2 mL) containing enzyme (26 μM), either [^{14}C]Val (90 μM, 280 Ci/mol) or [^{14}C]Ile (80 μM, 342 Ci/mol), ATP (1 mM), inorganic pyrophosphatase (5 units/mL) in the standard, pH 7.78, buffer was chilled and desalted on a 1 × 10 column containing Sephadex G-25 (fine) equilibrated with the standard, pH 5.87, buffer (without $MgCl_2$) at 4 °C.

Isoleucylation of tRNA. The conventional procedure as described by Fersht and Kaethner (1976a) was employed.

The ATP pyrophosphatase activity of IRS in the presence of valine and tRNA was measured by incubating [γ-^{32}P]ATP (1.83 mM), inorganic pyrophosphatase (4 units/mL), valine, and IRS (as indicated in the tables) in the standard buffers. Aliquots were periodically withdrawn and assayed for [^{32}P]-orthophosphate release by adding to a suspension (200 μL) of charcoal (1%) in 0.5 N HCl and potassium phosphate (25 mg/mL), and, after centrifugation, assaying the radioactivity in the supernatant by either scintillation or Čerenkov counting (cf. Baldwin and Berg, 1966).

Attempts to Detect a Steady-State Concentration of [^{14}C]Val-$tRNA^{Ile}$ During the ATP Pyrophosphatase Reaction. (a) 25 °C Complete Aminoacylation Mixture. One syringe of the pulsed quenched flow apparatus contained [^{14}C]Val (2.04 mM, specific activity = 5 mCi/mmol), $tRNA^{Ile}$ (14 μM), and isoleucyl-tRNA synthetase (11 μM) in the standard buffers. The other syringe contained ATP (5 mM) in the same buffer. Aliquots (292 μL) of the solutions were automatically mixed and quenched with trichloroacetic acid (5%) containing D,L-valine, and the precipitates were collected on Whatman GF/C filters. Blank experiments were performed omitting the ATP.

(b) At 0 °C, Using Preformed IRS·[^{14}C]Val-AMP. The enzyme-bound complex was prepared as described earlier but using the cacodylate buffer at pH 6.85. A solution of the complex (3 μM) and tRNA (3.4–2.8 μM $tRNA^{Ile}$) was incubated at 0 °C in this buffer (with 10 units/mL alkaline phosphatase). Aliquots (50 μL) were quenched with trichloroacetic acid at 15-s time intervals and the precipitates were collected on Whatman GF/C filters.

The tRNA-stimulated hydrolysis of IRS·Val-[^{32}P]AMP (1 μM) was measured under the same conditions by the method of Baldwin and Berg (1966).

(c) At 25 °C, Using Preformed IRS·[^{14}C]Val-AMP. One syringe of the pulsed quenched-flow apparatus contained IRS·[^{14}C]Val-AMP (0.8 μM) in a dilute, pH 5.87, buffer (10 mM). The other contained $tRNA^{Ile}$ (5 μM) in a concentrated Tris-Cl buffer (288 mM). The solutions were automatically mixed and quenched as in a.

The IRS-Catalyzed Hydrolysis of [^{14}C]Val-$tRNA^{Ile}$. One syringe of the quenched-flow apparatus contained IRS (2.5 μM) in the Tris-Cl buffer (288 mM, pH 7.78). The other contained [^{14}C]Val-$tRNA^{Ile}$ (0.05 μM, either fractionated or unfractionated) in 10 mM $MgCl_2$. The solutions were automatically mixed and quenched as above. The procedure was repeated using the IRS incubated with Ile (1 mM), ATP (5 mM), and inorganic pyrophosphatase (0.5 unit/mL) to form IRS·Ile-AMP.

Experiments on the Relative Stabilities of IRS·Ile, IRS·Val, IRS·Ile-AMP and IRS·Val-AMP. (a) Binding of Valine. The equilibrium-dialysis method was used as described previously (Fersht and Kaethner, 1976a), except that the competitive inhibition of the binding of [^{14}C]Ile by valine was measured under the standard conditions at pH 7.78 with [^{14}C]Ile (7 μM), valine (0.25–2 mM), and enzyme (6.4 μM).

(b) Hydrolysis of IRS·Ile-AMP and IRS·Val-AMP. The rates of hydrolysis of the complexes (~2 μM) at 25 °C in the standard, pH 7.78, buffer were determined by the retention of the enzyme-bound aminoacyl adenylate on nitrocellulose filters (Fersht, 1975). The effects of added Ile (in the presence of alkaline phosphatase (3 units/mL) to remove any residual ATP) and added Ile and ATP (in the presence of added inorganic pyrophosphatase (1 unit/mL)) were determined. In some experiments with IRS·[^{14}C]Val-AMP, VRS (3.6 μM) was added to trap any [^{14}C]Val-AMP that was expelled into solution.

(c) Rate of Dissociation of [^{14}C]Val-AMP from IRS·[^{14}C]Val-AMP. The [^{14}C]Val-AMP that dissociated from the enzyme-bound complex on the addition of Ile or Ile and ATP was measured by trapping with valyl-tRNA synthetase and then transferring the [^{14}C]Val to tRNA. A solution containing IRS·[^{14}C]Val-AMP (2 μM) and valyl-tRNA

TABLE I: Comparison of Valine-Stimulated ATP Pyrophosphatase Activity with Isoleucylation Activity of Isoleucyl-tRNA Synthetase.

Reaction	pH	Temp (°C)	k_{obsd}[a] (s^{-1})	K_M (Amino Acid) (μM)
ATP pyrophosphatase[b]	7.78	25	1.2	500
	6.85	25	0.5	
	5.87	25	0.3	
	6.85	0	0.02	
Ile-tRNA[c] synthesis	7.78	25	1.2	5.7
	5.87	25	0.3	
	6.85	0	0.02	

[a] Rate/[enzyme], for 14 μM $tRNA^{Ile}$, 1.9 mM ATP (saturating) and either 5 mM valine or 50 μM isoleucine. Rate constants are derived from steady-state kinetics. [b] 5 mM valine. [c] 50 μM isoleucine.

TABLE II: Comparison of Valine- and Isoleucine-Stimulated Pyrophosphate Exchange Activities.[a]

Amino Acid	$tRNA^{Ile}$ (μM)	k[b] (s^{-1})	K_M (Amino Acid) (μM)
Isoleucine (100 μM)	0	48	4[c] (5[d])
	16	34	6[c]
Valine (5 mM)	0	31	(390[d])
	16	21	

[a] 25 °C, pH 7.78, Tris, 10 mM $MgCl_2$, 2 mM [^{32}P]pyrophosphate, 1.83 mM ATP. [b] (Initial rate of incorporation of ^{32}P into ATP)/[enzyme]. [c] Fersht and Kaethner (1976a). [d] Bergmann et al. (1961) (*E. coli* B).

synthetase (3.6 μM) was incubated in the standard, pH 7.78, buffer with various concentrations of isoleucine (and 0.3 unit/mL alkaline phosphatase) or isoleucine and ATP. Aliquots (20 μL) were periodically taken, added to a solution of $tRNA^{Val}$ (23 μM, 50 μL), and immediately (~2 s) quenched with cold trichloroacetic acid. The precipitate was collected on a nitrocellulose filter and, after washing and drying, the [^{14}C]Val-$tRNA^{Val}$ was assayed by scintillation spectrophotometry.

(d) Fate of the [^{14}C]Val-AMP on the Addition of tRNA to IRS·[^{14}C]Val-AMP. Attempts to transfer [^{14}C]Val from the IRS·[^{14}C]Val-AMP complex to unfractionated tRNA (5 μM $tRNA^{Ile}$, 27.5 μM $tRNA^{Val}$) were made at pH 7.78 by: (a) adding the complex (2 μM) to the tRNA and quenching with trichloroacetic acid to collect any charged material; (b) repetition of the above but immediately adding valyl-tRNA synthetase (3.3 μM) after mixing the complex with tRNA in order to trap any [^{14}C]Val-AMP that may be released (in this case, valine (100 μM) and alkaline phosphatase (0.3 units/mL) were added to suppress any charging of $tRNA^{Val}$ by the VRS using residual ATP and [^{14}C]Val that is released by the hydrolysis of [^{14}C]Val-AMP); (c) as a but, instead of being quenched, the mixture was filtered through nitrocellulose disks to detect any remaining IRS·[^{14}C]Val-AMP.

Results

Where possible, numerical data are presented in the form "mean ± standard error".

Valine-Stimulated ATP Pyrophosphatase Activity of IRS in the Presence of tRNA and ATP. Over a wide range of conditions, the turnover number for the hydrolysis of ATP to AMP catalyzed by the isoleucyl-tRNA synthetase in the presence of valine and tRNA is identical to that for the isoleucylation of tRNA catalyzed by the enzyme when the valine is replaced by [^{14}C]Ile (Table I). (The rate-determining step in isoleucylation is the transfer of the isoleucine from IRS·Ile-AMP to the tRNA or a step immediately preceding it.) At pH 6.85 and 0 °C, the turnover number for the ATP pyrophosphatase reaction is the same as the first-order rate constant for the release of [^{32}P]AMP on the addition of tRNA to IRS·Val-[^{32}P]AMP.

The IRS·Val-AMP complex is certainly formed during the editing reaction: in the presence of valine, tRNA, and 2 mM [^{32}P]pyrophosphate at pH 7.78, the enzyme catalyzes the exchange of pyrophosphate into ATP considerably faster than the overall ATP pyrophosphatase activity (Table II). The K_M for valine (500 μM) is similar to that for the K_I for the competitive inhibition by valine of the isoleucylation of tRNA (400μM, Kondo and Woese, 1969; 380 μM, Bergmann et al., 1961).

The observations so far would appear to be consistent with a scheme in which the enzyme rapidly forms an IRS·Val-AMP complex and then, at saturating substrate concentrations, transfers the valyl moiety to $tRNA^{Ile}$ in the rate-determining step which is then followed by the rapid hydrolysis of the Val-$tRNA^{Ile}$. But, as shown next, there is an inconsistency in the experimental evidence.

Search for a Steady-State Concentration of [^{14}C]Val-tRNA. If the specificity mechanism involves the formation of Val-$tRNA^{Ile}$ followed by its enzyme-catalyzed hydrolysis, as in eq 1

$$\text{IRS·tRNA} \xrightarrow{\text{ATP}} \text{IRS·tRNA·Val-AMP} \xrightarrow{k} \text{IRS-Val-tRNA} \xrightarrow{k_h} \text{IRS} + \text{Val} + \text{tRNA} \quad (1)$$

then there will be a steady-state concentration of Val-tRNA given by:

$$[\text{Val-tRNA}^{Ile}]_{SS} = (k/k_h)[\text{IRS·tRNA·Val-AMP}] \quad (2)$$

(If the Val-$tRNA^{Ile}$ dissociates from the enzyme, the steady-state concentration will be greater than that predicted by eq 2.)

Attempts to detect this steady-state concentration of Val-$tRNA^{Ile}$ using either the complete aminoacylation mixture or by adding tRNA to IRS·[^{14}C]Val-AMP at 25 °C and pH 7.78 in the quenched-flow apparatus show that the level of Val-$tRNA^{Ile}$ formed is very low. The amount of [^{14}C]Val-$tRNA^{Ile}$ formed in both cases is less than 0.8% of either the IRS or the IRS·[^{14}C]Val-AMP initially present. The ratio of k_h/k would have to be greater than 125 to account for this; that is, k_h must be greater than 150 s^{-1}.

Similarly, experiments at 0 °C using either cacodylate buffer (pH 6.87) or Bistris-Cl (pH 6.23) led to a transient transfer of less than 1% of the radioactivity from IRS·[^{14}C]Val-AMP to tRNA (over the time range 7 s to several minutes).

Direct Measurement of the IRS-Catalyzed Hydrolysis of [^{14}C]Val-$tRNA^{Ile}$. [^{14}C]Val-$tRNA^{Ile}$ was prepared by the misacylation of partially purified $tRNA^{Ile}$ or unfractionated tRNA in which all the tRNA species other than $tRNA^{Ile}$ had been periodate oxidized. On mixing with excess enzyme at 25 °C and pH 7.78, both samples are hydrolyzed in a biphasic manner (Figure 1). Sixty percent is rapidly hydrolyzed with a first-order rate constant of 10 s^{-1}, while the remainder more

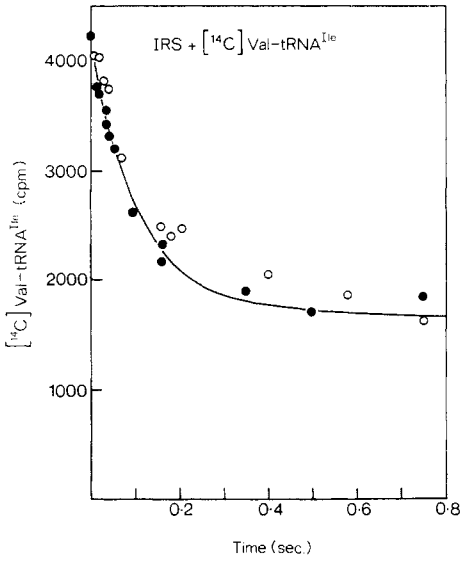

FIGURE 1: IRS-catalyzed hydrolysis of Val-tRNA^{Ile} at pH 7.78, 25 °C, and 10 mM $MgCl_2$. (●) Fractionated Val-tRNA^{Ile}; (○) Val-tRNA^{Ile} + oxidized tRNA (see text).

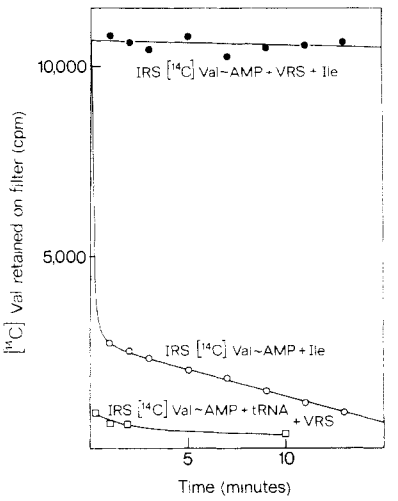

FIGURE 2: Stability of IRS·Val-AMP. (○) Addition of isoleucine (500 μM) to IRS·[^{14}C]Val-AMP and filtration through nitrocellulose filters; (●) as before, but the addition of VRS with the isoleucine (the [^{14}C]Val is retained as VRS·[^{14}C]Val-AMP); (□) addition of tRNA to IRS·[^{14}C]Val-AMP, followed by the addition of VRS. Aliquots were quenched with Cl_3CCOOH to trap any [^{14}C]Val-tRNA formed (see text).

slowly with a half-life of seconds. A similar biphasic behavior at 4 °C and pH 7 was reported by Eldred and Schimmel (1972). The reason for this heterogeneity is not known.

The rate constant of 10 s^{-1} for the fast phase is well below the value of 150 s^{-1}, which is required in eq 1 to account for the low concentration of Val-tRNA^{Ile}.

Addition of isoleucine, ATP, and inorganic pyrophosphatase to transform the IRS into IRS·Ile-AMP decreases the rate constant for the fast phase to 3.3 s^{-1}. This decrease is similar to that noted for the inhibition of the hydrolysis of Ile-tRNA^{Ile} (Schreier and Schimmel, 1972).

The rate constant for the hydrolysis of Ile-tRNA^{Ile} catalyzed by IRS at saturating concentrations is 0.014 s^{-1} at 25 °C and pH 7.78.

Stabilities and Dissociation Rates of IRS·Ile-AMP and IRS·Val-AMP. Binding of Valine vs. Isoleucine. The binding of valine was determined from the competition of the binding of [^{14}C]Ile using equilibrium dialysis. The equations for mass balance may be rearranged to give:

$$\frac{[ILE][IRS]}{[IRS\cdot Ile]} - [Ile] = K_{Ile} + [Val]K_{Ile}/K_{Val} \qquad (3)$$

where K_{Ile} and K_{Val} are the dissociation constants of the IRS·Ile and IRS·Val complexes. K_{Ile} was determined to be 8.1 ± 1 μM and K_{Val}/K_{Ile} to be 109 ± 11. These values may be compared with the previously determined K_{Ile}, 6.7 ± 0.6 μM (Fersht and Kaethner, 1976a) and K_{Ile} = 4.35 μM, K_{Val}/K_{Ile} = 131 for the IRS from *E. coli* MRE 600 (Flossdorf and Kula, 1973). The binding energy of the additional methylene group is at least 2.8 kcal (12 kJ)/mol.

Stability of IRS·[^{14}C]Ile-AMP and IRS·[^{14}C]Val-AMP. At pH 7.78, 10 mM $MgCl_2$, and 25 °C, both the IRS·Ile-AMP and IRS·Val-AMP complexes hydrolyze with a first-order rate constant of 7 × 10^{-4} s^{-1}. The complexes are more stable than the free aminoacyl adenylates, which hydrolyze at 2.9 × 10^{-3} s^{-1} and 3.7 × 10^{-3} s^{-1}, respectively (unpublished data). Whereas the addition of isoleucine (500 μM) increased the hydrolysis rate of IRS·[^{14}C]Ile-AMP to only 10 × 10^{-4} s^{-1} and the addition of isoleucine and ATP to 17 × 10^{-4} s^{-1}, the addition of isoleucine (500 μM) to IRS·[^{14}C]Val-AMP caused 80% of the Val-AMP bound to the enzyme to be lost within a minute, while the remainder hydrolyzed at a rate constant of ca. 12.5 × 10^{-4} s^{-1} (Figure 2).

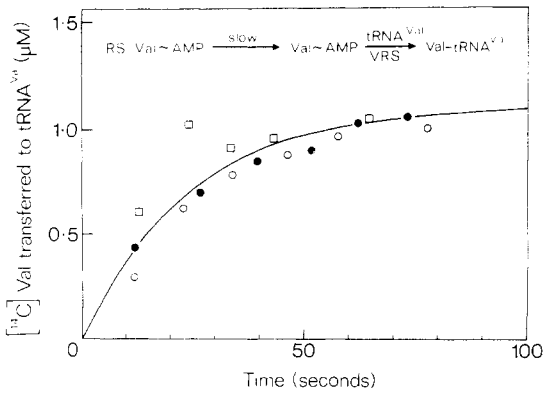

FIGURE 3: Dissociation rate of IRS·[^{14}C]Val-AMP. The rate of dissociation of the [^{14}C]Val-AMP was determined by "chasing off" with either isoleucine (500 μM, ●) or isoleucine (500 μM) and ATP (2.5 mM, □), or with no added isoleucine (○), and assaying the dissociated [^{14}C]Val-AMP by transferring the [^{14}C]Val to tRNA^{Val} via the VRS (see text).

Fate of the [^{14}C]Val-AMP. The Val-AMP which was rapidly lost was shown by trapping with valyl-tRNA synthetase to be expelled into solution, rather than hydrolyzed enzymatically. The addition of 2 equiv of VRS with the isoleucine to the IRS·[^{14}C]Val-AMP complex led to the transfer of the [^{14}C]Val-AMP to the valyl-tRNA synthetase, as shown by the retention of all the radioactivity on nitrocellulose filters (Figure 2) or by the formation of [^{14}C]Val-tRNA^{Val} on the subsequent addition of tRNA.

(The biphasic loss of [^{14}C]Val-AMP on the addition of isoleucine to IRS·[^{14}C]Val-AMP complex is presumably due to a relatively rapid equilibration between the complex and IRS·Ile, followed by a slower decrease as the [^{14}C]Val-AMP hydrolyzes both in solution and on the enzyme.)

Dissociation Rate of IRS·[^{14}C]Val-AMP. This was measured by using the valyl-tRNA synthetase to trap the [^{14}C]Val-AMP that dissociated from the IRS by transferring the [^{14}C]Val to tRNA^{Val}. The rate constant for the dissociation is about 0.04 s^{-1} at 20 °C (Figure 3). (It should be noted that

this rate constant is considerably slower than the rate of transfer of Ile from IRS·Ile-AMP to tRNA (1.5 s^{-1}, Fersht and Kaethner, 1976a) or the turnover number for the valine-stimulated ATP pyrophosphate activity.)

Fate of the [^{14}C]Val-AMP on the Addition of tRNA to IRS·[^{14}C]Val-AMP. The addition of tRNA to IRS·[^{14}C]Val-AMP at 25 °C led to no detectable acid-precipitable counts. The addition of tRNA immediately followed by the addition of valyl-tRNA synthetase to trap any [^{14}C]Val-AMP expelled into solution again gave no acid-precipitable counts or retention by nitrocellulose filters. This confirms the proposal by Baldwin and Berg (1966) that the valyl adenylate is (indirectly) enzymatically hydrolyzed, rather than expelled into solution, since in the absence of enzyme the Val-AMP has a half-life of 3 min under the reaction conditions.

Discussion

The specificity mechanism for the rejection of threonine by the VRS from *B. stearothermophilus* involves the formation of the enzyme-bound threonyl adenylate complex followed by the transfer of the threonine to $tRNA^{Val}$ and the subsequent rapid deacylation of the mischarged tRNA. The specificity mechanism for the rejection of valine by the IRS from *E. coli* appears, at first sight, to be similar. Pyrophosphate-exchange kinetics in the presence of tRNA show that the IRS·Val-AMP·tRNA complex is rapidly formed (Table II). The turnover number for the ATP pyrophosphatase activity is consistent with the rate-determining transfer of the Val from the adenylate to the tRNA, since the rate is very similar to that for the transfer of Ile from IRS·Ile-AMP to $tRNA^{Ile}$. But whereas in the case of the VRS and threonine the mischarged tRNA may be trapped in rapid-quenching experiments and its concentration found to be consistent with the independently measured rate constants for its formation and subsequent enzyme-catalyzed hydrolysis, the same is not true for the IRS and valine. On mixing the preformed IRS·[^{14}C]Val-AMP complex with tRNA, less than 0.8% of the radioactivity is transferred to the tRNA. A similar result is found on mixing the IRS with tRNA, ATP, and [^{14}C]Val. This requires that the rate constant for the enzyme-catalyzed hydrolysis of the Val-$tRNA^{Ile}$ be at least 125 times faster than the rate constant for the transfer of the amino acid to the tRNA, i.e., 150 s^{-1} for a transfer rate constant of 1.2 s^{-1}. This hydrolytic rate constant could well be a considerable underestimate of the actual value, since the figure of 0.8% is at the level of the impurities in the [^{14}C]Val and the actual transfer of [^{14}C]Val may be below this. Yet, the observed rate constant for the deacylation is only 10 s^{-1}.

There are several reasons that could account for this discrepancy. (a) The deacylation rate of tRNA that has been artificially misacylated at high temperature may be artifactually low due to the tRNA being partly denatured. We feel this is unlikely. (b) The enzyme might be in a different, hydrolytically active, conformation during the transfer of the amino acid from the adenylate to the tRNA.
For example,

$$\text{IRS} \longrightarrow \text{IRS*·Val-AMP·tRNA} \xrightarrow{1.2\ s^{-1}} \text{IRS*·Val-tRNA} \xrightarrow{150\ s^{-1}} \text{Val} + \text{tRNA} \quad (4)$$

Whereas the rate-determining step in the independently measured deacylation rate could be a conformational change to give the active conformation.

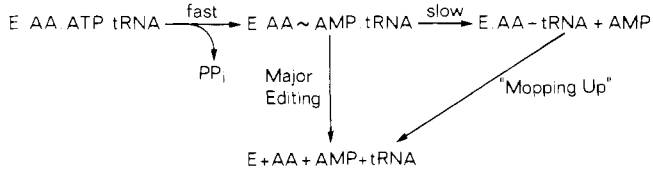

FIGURE 4: A possible two-step editing mechanism.

$$\text{IRS} \xrightarrow{\text{Val-tRNA}} \text{IRS·Val-tRNA} \xrightarrow{10\ s^{-1}} \text{IRS*·Val-tRNA} \xrightarrow{150\ s^{-1}} \text{Val} + \text{tRNA} \quad (5)$$

(c) Alternatively, there may be a different specificity mechanism in which the Val-AMP is hydrolyzed *before* the transfer of the Val to the tRNA. The hydrolytic activity of the IRS towards Val-tRNA would then be just a double check to mop up any misacylated tRNA that leaks through the first editing step (Figure 4).

For example, the rate-determining step could be a conformational change to give an activated intermediate as in eq 6 or 7.

$$\begin{array}{ccc} \text{IRS·Val-AMP·tRNA} & & \\ \downarrow 1.2\ s^{-1} & & \\ \text{IRS*·Val-AMP·tRNA} & \xrightarrow{\text{slow}} & \text{IRS·Val-tRNA} \\ \downarrow \text{fast} & \swarrow 10\ s^{-1} & \\ \text{IRS} + \text{Val} + \text{AMP} + \text{tRNA} & & \end{array} \quad (6)$$

In the case of valine the hydrolytic step would be faster than the transfer, whereas for isoleucine the transfer step would be faster than the hydrolytic.

$$\begin{array}{ccc} \text{IRS·Ile-AMP·tRNA} & & \\ \downarrow 1.2\ s^{-1} & & \\ \text{IRS*·Ile-AMP·tRNA} & \xrightarrow{\text{fast}} & \text{IRS·Ile-tRNA} \\ \downarrow \text{slow} & & \\ \text{IRS} + \text{Ile} + \text{AMP} + \text{tRNA} & & \end{array} \quad (7)$$

It is not possible to distinguish between mechanisms b and c on the basis of the present data. However, it is possible to rule out the "kinetic proofreading" mechanism of Hopfield (1974). In this, the Val-AMP is not enzymically hydrolyzed but diffuses into the solution, where it hydrolyzes, faster than the amino acid is transferred to the tRNA. However, the results of this study and that of Baldwin and Berg (1966) show that the hydrolysis *is* catalyzed by the enzyme; the half-life of Val-AMP in solution at pH 7.8 and 25 °C is 3 min, but on addition of the tRNA to IRS·Val-AMP, the adenylate is destroyed in seconds. Also, the dissociation rate of the Val-AMP from the IRS is very slow, only 0.04 s^{-1}. The evidence recently adduced in favor of the kinetic proofreading scheme (Hopfield et al., 1976) is also consistent with the mechanisms proposed in this study.

von der Haar and Cramer (1976) very recently reported turnover numbers of 0.03 and 0.16 s^{-1}, respectively, for the hydrolysis of Ile-$tRNA^{Ile}$ and Val-$tRNA^{Ile}$ catalyzed by the IRS from yeast at 36 °C. These values may be compared with 0.014 and 10 s^{-1} at 25 °C for the reactions of the *E. coli* enzyme found in this study. The misacylated tRNA is deacylated 60 times more slowly by the yeast enzyme, despite the higher temperature. In view of our results, it seems very unlikely that this very low hydrolytic rate constitutes the major editing step.

Thus, the hydrolytic rates measured for the yeast IRS, as well as for the IRS from *E. coli*, represent either a secondary "mopping-up" editing step, or artifactually low values due to the enzyme being in a less active conformation. The only system in which it has been conclusively demonstrated that a large fraction of the "wrong" amino acid is transferred to the tRNA and then deacylated in the major editing step is that of the rejection of threonine by the VRS from *B. stearothermophilus* (Fersht and Kaethner, 1976b).

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