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Role of the β-Phosphate–γ-Phosphate Interchange Reaction of Adenosine Triphosphate in Amino Acid Discrimination by Valyl- and Methionyl-tRNA Synthetases from *Escherichia coli*†

Linda Tombras Smith and Mildred Cohn*

ABSTRACT: Aminoacyl-tRNA synthetases catalyze a novel amino acid dependent interchange reaction in which the β- and γ-phosphates of ATP are interchanged independent of the ATP–PP_i exchange reaction. This reaction is designated the direct interchange reaction. To assess the role of the direct interchange reaction in preventing misaminoacylation of tRNA, the reactions catalyzed by Val- and Met-tRNA synthetases from *Escherichia coli* were investigated with cognate and noncognate amino acids as substrates, and the relative contributions of the ATP–PP_i exchange reaction and the direct interchange reaction to the overall rate of interchange were evaluated. The kinetic parameters (with respect to amino acid) of the total interchange reaction were compared to those of the exchange reaction with cognate and noncognate amino acids. The latter included Thr, Ile, or α-aminobutyric acid for Val-tRNA synthetase and DL-homocysteine for Met-tRNA synthetase. In the interchange reaction, V_{max} values with noncognate amino acids were 4–6-fold higher than those with the cognate amino acid. Furthermore, the ratio V_{max}(interchange)/V_{max}(exchange) was 8–30-fold greater for the noncognate than the cognate amino acids. The addition of 10 μM to 1 mM PP_i increased the rate of total interchange with cognate amino acids 3–18-fold. In contrast, with noncognate amino acids, the total interchange rates increased slightly (less than 20%) at 10 μM PP_i and in fact decreased at higher PP_i concentrations. Thus, interchange with cognate amino acids is predominantly exchange mediated. In contrast, with noncognate amino acids, the exchange reaction does not play a significant role in interchange, and interchange results predominantly from the direct interchange reaction. The striking difference in the behavior of cognate and noncognate amino acids even under more physiological conditions (in the presence of appropriate amounts of tRNA, inorganic pyrophosphatase, and P_i) led to the conclusion that the direct interchange reaction provides a contributory mechanism for preventing the aminoacylation of tRNA by noncognate amino acids.

Most aminoacyl-tRNA synthetases carry out an amino acid dependent ATP–PP_i exchange as well as the aminoacylation of tRNA (Kisselev & Favorova, 1974). As shown in a previous report (Rossomando et al., 1979), Val- and Met-tRNA synthetases of *E. coli* can carry out an amino acid dependent reaction designated the direct interchange reaction with ATPβS[1] as substrate in which the β- and γ-P of ATPβS are interchanged without dissociation of SPP_i from the enzyme. This reaction was shown to be distinct from an ATPβS–SPP_i exchange reaction. As depicted in Scheme I in the form of minimal sequences, the total observed interchange of β-P and γ-P of ATP may be formulated as the sum of contributions from the direct interchange reaction (reaction 1) and the exchange-mediated reaction (reaction 2a). The exchange-mediated interchange reaction is identical with the ATP–PP_i exchange reaction in all respects except that the concentration of PP_i never exceeds the enzyme concentration and only half of the exchanges can be detected as net interchange (see reactions 2a and 2b). By use of ATPβS, it was possible to reduce greatly the contribution, if any, of the exchange-mediated reaction to the total interchange since the exchange rate is so drastically reduced with the thio analogue as substrate that only the direct interchange mechanism is responsible for the observed interchange reaction. The very slow exchange rate and the opposite stereospecificities of the (*R*)- and (*S*)-ATPβS diastereomers in the interchange and aminoacylation reactions clearly demonstrated that an interchange reaction distinct from the exchange-mediated reaction does exist with this substrate.

The present study was undertaken to determine the contributions of the exchange-mediated and direct interchange reactions to the observed interchange with ATP, the normal

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[1] Abbreviations used: AA-AMP, aminoacyl adenylate; Abu, α-aminobutyric acid; ATPβS, adenosine 5′-*O*-(2-thiotriphosphate); BSA, bovine serum albumin; Hepes, *N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Hcy, homocysteine; PEI–cellulose, poly(ethylenimine)–cellulose.

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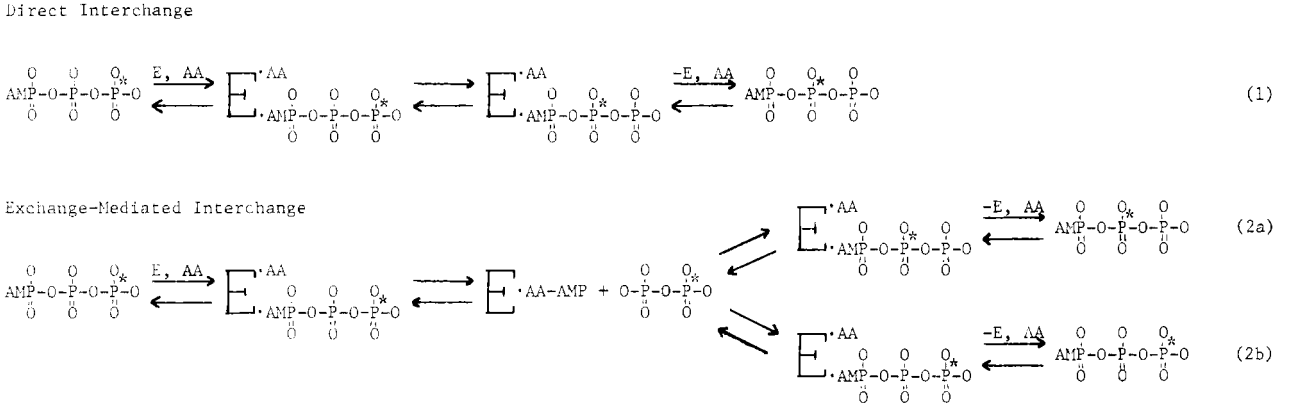
Scheme I



substrate for the reaction. On the basis of the earlier experiments, it was suggested that a physiological role for the direct interchange reaction might be prevention of misincorporation of noncognate amino acids in protein synthesis in such a way that ATP is merely recycled, never achieving aminoacylation of tRNA, whereas the cognate amino acid undergoes the normal acylation reaction to form products. In the case of various reactions catalyzed by Val-tRNA synthetase, the K_m and V_{max} values for the cognate amino acid, Val, are compared to those of the noncognate naturally occurring amino acids Thr and Ile and the unnatural amino acid α-aminobutyric acid (Abu). The reactions catalyzed by Met-tRNA synthetase are investigated with Met and with the noncognate amino acid homocysteine (Hcy) as substrates. An attempt is made to estimate the contribution of the direct interchange reaction to the fidelity of tRNA aminoacylation under physiological conditions relative to other error-correcting mechanisms, in particular hydrolysis of noncognate aminoacyl adenylate and aminoacyl-tRNA.

Materials and Methods

Materials. *E. coli* (K-12) was purchased from Grain Processing Corp. Val, Thr, Ile, Met, DL-Hcy, and inorganic pyrophosphatase (yeast) were obtained from Sigma Chemical Co. α-Aminobutyric acid was from Aldrich Chemical Co. Hexokinase (yeast) was obtained from Worthington. $tRNA^{fMet}$ was purchased from Plenum Scientific, and $tRNA^{Val}$ was a generous gift from B. Roe of Kent State University. Spermidine was purchased from Calbiochem. [γ-^{32}P]ATP ($\sim$20 Ci/mmol), [^{32}P]PP_i ($\sim$1 Ci/mmol), and [^{33}P]P_i (0.1 Ci/mmol) were obtained from New England Nuclear, and [8-^{14}C]ATP (26 mCi/mmol) was obtained from Schwarz/Mann. PEI–cellulose (F-5504) plastic sheets were purchased from Merck, and Instabray scintillation solution was from Yorktown Research.

The Val- and Met-tRNA synthetases were purified as described previously (Rossomando et al., 1979). Interchange was followed by the production of [β-^{32}P]ATP from [γ-^{32}P]ATP under the following conditions: 40 mM Hepes, pH 8.0, 0.2 mg/mL BSA, 6 mM 2-mercaptoethanol, 4 mM magnesium acetate, 1 mM [γ-^{32}P]ATP (10^7 cpm/μmol), and from 2 to 40 mM amino acid; total volume 200 μL and T = 30 °C. The course of the reaction, initiated by addition of enzyme, was followed by removing 20-μL aliquots at chosen time intervals. The reaction was stopped by the addition of a 2-μL solution containing 4 μg of hexokinase and 40 mM glucose. The amount of hexokinase was enough to convert well over 90% of the ATP in the reaction mixture to ADP within 30 s. After all of the ATP was converted to ADP, the components were separated by thin-layer chromatography. A 10-μL aliquot of the reaction mixture was spotted on a PEI–cellulose plate and developed for 30 min in 0.75 M KH_2PO_4, pH 3.5. The ADP spot, identified by fluorescence, was cut out and counted for ^{32}P in 5 mL of Instabray with a Beckmann LS-100C multichannel liquid scintillation counter.

Exchange was monitored by the appearance of ^{32}P in unlabeled ATP in a 50-μL reaction volume containing 1 mM Mg[^{32}P]PP_i (10^7 cpm/μmol); all other conditions were the same as those in the interchange reaction. The reactions were stopped after 15 min at 30 °C with 5 μL (1 μg) of pyrophosphatase. A 10-μL aliquot of the reaction mixture was spotted on a PEI–cellulose plate for thin-layer chromatography and developed in 0.38 M KH_2PO_4, pH 3.5, and the ATP spot was removed and counted for ^{32}P as described above.

ATP breakdown was followed under the same conditions as interchange except that [^{14}C]ATP (10^7 cpm/μmol) was used instead of [γ-^{32}P]ATP, and no hexokinase was added. The reaction was stopped by spotting 10 μL on a PEI–cellulose sheet. Cold carrier AMP (5 nmol) was added to each spot, the sheet was developed in 0.75 M KH_2PO_4, pH 3.5, and the ^{14}C in the AMP spot was counted. In some experiments, ATP breakdown was followed with [γ-^{32}P]ATP, the reaction was stopped by spotting 10 μL on a PEI–cellulose plate, and the sheet was developed in 1 M KH_2PO_4, pH 3.5. The PP_i spot was cut out and counted for ^{32}P. The R_f of PP_i was determined with authentic [^{32}P]PP_i. In experiments where both interchange and exchange were followed simultaneously with labeled [γ-^{32}P]ATP and unlabeled PP_i, the rate of exchange was monitored by the increase of ^{32}P in the PP_i spot on the PEI–cellulose plate. The [ATP] was 1 mM in all reaction mixtures; both synthetases are saturated with ATP (K_m < 0.2 mM) in the presence of their cognate amino acids, and it was assumed that 1 mM ATP is also sufficient for saturation with noncognate amino acids.

The rates of interchange, exchange, and breakdown were determined simultaneously in the presence of inorganic pyrophosphatase by using thin-layer chromatography. The following conditions were used: 40 mM Hepes, pH 8.0, 0.2 mg/mL BSA, 6 mM 2-mercaptoethanol, 6 mM magnesium acetate, 1 mM [γ-^{32}P]ATP (10^7 cpm/μmol), 0.5 mM sperimidine, 5 mM [^{33}P]P_i (1.5×10^8 cpm/μmol), 5 mg/mL yeast inorganic pyrophosphatase, 2 μM $tRNA^{fMet}$, either 40 mM Hcy or 2 mM Met, and 4 mg/mL Met-tRNA synthetase. The ratio of inorganic pyrophosphatase to Met-tRNA synthetase was adjusted so that it would be similar to that in the bacterial cell by using the value (Josse, 1966) of the specific activity of inorganic pyrophosphatase in crude extracts of *E. coli* which is constant and independent of strain. At each time point, 10 μL was removed, spotted directly on a PEI–cellulose plate, and developed with the 0.75 M KH_2PO_4 solvent, and the ATP spot

Table I: Kinetic Constants of the Exchange and Interchange Reactions Catalyzed by Val- and Met-tRNA Synthetases[a]

		exchange		interchange		
enzyme	amino acid	K_m (mM)	I, V_{max} (μmol min^{-1} mg^{-1})	K_m (mM)	II, V_{max} (μmol min^{-1} mg^{-1})	ratio I/II
Val-tRNA synthetase	Val	0.08	21.6	0.02	0.20	108
	Thr	10	5.8	5.3	1.15	5.0
	Ile	15	3.3	13	0.94	3.5
	Abu	2.2	2.9	2.4	0.80	3.6
Met-tRNA synthetase	Met	0.026	50.5	0.008	1.80	28
	DL-Hcy	48[b]	32.6	26[b]	9.10	3.6

[a] The exchange reaction was carried out at 30 °C in 40 mM Hepes, pH 8.0, 0.2 mg/mL BSA, 6 mM 2-mercaptoethanol, 5 mM magnesium acetate, 1 mM ATP, and 1 mM [^{32}P]PP$_i$. The interchange reaction was carried out under the same conditions except that PP$_i$ was omitted and 4 mM magnesium acetate and 1 mM [γ-^{32}P]ATP were used. The K_m values listed refer to the amino acids. [b] The K_m for L-Hcy is probably half the value listed; since D-Met is not a substrate, D-Hcy also is probably not a substrate.

was cut out and counted for (1) ^{33}P incorporation (representing exchange) and (2) ^{32}P loss (representing breakdown plus exchange). The breakdown rate is the difference between the two rates. In order to monitor interchange, another 20 μL was removed at each time point, reactions were stopped with hexokinase, and ^{32}P incorporation into the β-P of ATP was followed as described above. ^{33}P was counted in the ^{3}H channel of the liquid scintillation counter, and ^{32}P was counted in the ^{32}P channel adjusted so that less than 0.1% of the ^{33}P counts were detected in the ^{32}P channel. With this adjustment, resolution is not complete; 75% of the ^{32}P counts were detected in the ^{32}P channel, and about 5% of the total ^{32}P counts were still detected in the ^{3}H channel. Using the ^{3}H channel to count ^{33}P was possible because of quenching due to the fact that the isotope was absorbed to the PEI–cellulose plates. About 50% of the total ^{33}P counts were detected in the ^{3}H channel. Sample geometry was found to be important in these double-label experiments. For good reproducibility, 7-mL scintillation vials (Kimble Solvent Saver scintillation vials) were used, and samples were placed vertically in vials.

Results

Relative Contributions of Direct and Exchange-Mediated Interchange to the Observed Interchange Reaction

Kinetic Parameters of the Interchange and Exchange Reactions. The rates of interchange (γ-^{32}P $\rightleftarrows$ β-^{32}P) and exchange (ATP $\rightleftarrows$ [^{32}P]PP$_i$) catalyzed by Val-tRNA synthetase with Val, and three noncognate amino acids, Thr, Ile, and Abu, are compared in Table I. The Michaelis constants and the maximum velocities with respect to amino acid were calculated from double-reciprocal plots of initial velocity vs. amino acid concentration. Of the amino acids tested, Val is by far the best substrate, in terms of K_m and V_{max}, in the exchange reaction. The V_{max} values with noncognate amino acids range from 4 to 8 times lower than that for Val. On the other hand, in the interchange reaction, the V_{max} values with noncognate amino acids are from 4 to 6 times higher than that for Val. The ratio of the interchange to exchange rates increases by a factor of 20–30 for the noncognate amino acids relative to Val. If the interchange resulted solely from the exchange-mediated reaction, then the pattern of V_{max}'s for both exchange and interchange, i.e., Val > Thr > Ile > Abu, should be the same, since both interchange and exchange would reflect the same pathway. Consequently, the results support the conclusion that a direct interchange reaction occurs distinct from exchange. Since interchange rates with the noncognate amino acids are greater than with Val, the possibility that the interchange with the noncognate amino acids is due to a contamination with Val in those amino acid preparations is ruled out.

Similar experiments were conducted with another enzyme, Met-tRNA synthetase of *E. coli*. Table I shows that for Met as substrate the V_{max} is higher than that for DL-Hcy in the exchange reaction but 5 times lower than that for Hcy in the interchange reaction. The ratio of the interchange rate to the exchange rate with Hcy is increased about 8-fold relative to Met.

Before the increased V_{max} values of interchange with noncognate amino acids are ascribed to a major contribution of the direct interchange reaction, an alternative explanation must be eliminated. It remains to be demonstrated that under the conditions of the interchange experiments, i.e., no added PP$_i$, the exchange-mediated interchange rate for the noncognate amino acids is not greatly stimulated relative to that for cognate amino acids. A large differential stimulation of the exchange-mediated reaction would arise if the rate of its reverse reaction (E·AA-AMP + PP$_i$ $\rightleftarrows$ E + AA + ATP) is greatly increased in the presence of noncognate amino acids. Such an increase may be effected by (1) increasing the concentration of free PP$_i$ or (2) decreasing the K_m of PP$_i$. An increase in the free PP$_i$ concentration could occur by the following sequence of events. The noncognate aminoacyl adenylate (AA-AMP) could dissociate more readily from the synthetase than the cognate AA-AMP. As a consequence, since the free AA-AMP is far less stable to hydrolysis than the enzyme-bound species (Berg, 1958), the noncognate AA-AMP would tend to break down to amino acid and AMP. More importantly, PP$_i$ would also be released to the medium since PP$_i$ binds tightly to the enzyme only when AA-AMP is also bound (Santi et al., 1971; Blanquet et al., 1975). The net reaction would be ATP breakdown to AMP and PP$_i$, which can be measured experimentally. The increased PP$_i$ concentration would cause a net increase in the rate of the exchange reaction provided the enzyme remains saturated with AA-AMP. If the latter condition does not hold, due to a high rate of hydrolysis of the AA-AMP, then the effect of increased PP$_i$ concentration is counteracted. Thus, the reverse reaction and consequently the overall exchange reaction would again be slow or negligible. As the K_m for PP$_i$ decreases, the rate of exchange-mediated interchange approaches the rate of exchange since the reverse reaction of the exchange-mediated reaction under conditions of interchange experiments would occur closer to saturation with PP$_i$. Thus, a large difference in the measured K_m values of PP$_i$ between cognate and noncognate amino acids could cause an increase in the ratio of V_{max}(interchange)/V_{max}(exchange) with noncognate amino acids. An additional experimental approach bearing on the consequence of increased PP$_i$ concentration is to observe the effect of external PP$_i$ added at various levels to the reaction mixture on interchange rates with cognate and noncognate amino acids.

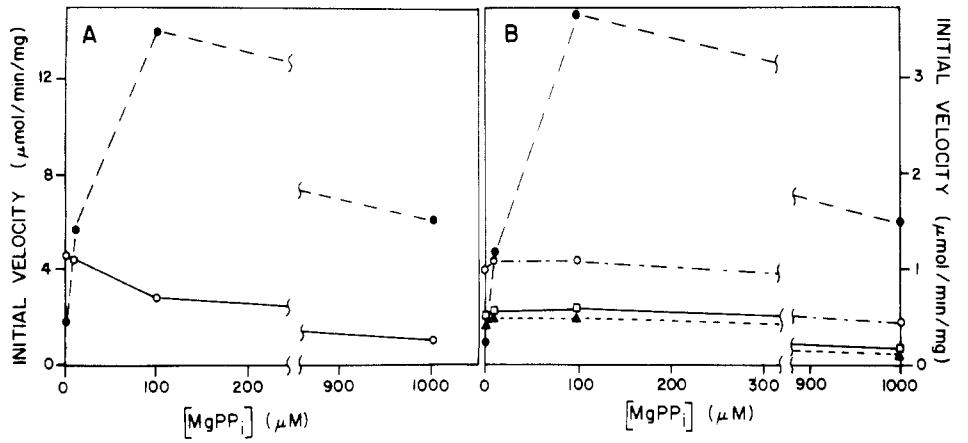


FIGURE 1: Effect of $MgPP_i$ on the rate of the interchange reaction. Rates of interchange were determined under the conditions described under Materials and Methods. (A) Met-tRNA synthetase: 2 mM Met (●---●); 40 mM Hcy (○—○). (B) Val-tRNA synthetase: 2 mM Val (●---●); 20 mM Thr (○···○); 10 mM Abu (□—□); 40 mM Ile (▲---▲).

Table II: Contribution of the Exchange Reaction to the Rate of Interchange[a]

enzyme	amino acid	concn (mM)	exchange rate (μmol min^{-1} mg^{-1})	^{32}P interchange (cpm)		obsd/ calcd
				obsd	calcd	
Val-tRNA synthetase	Val	2	25	8000	14000	0.6
	Thr	20	3.5	5500	4900	1.1
	Ile	40	0.98	1500	490	3.3
	Abu	10	1.2	2000	640	3.1
Met-tRNA synthetase	Met	2	52.5	3800	2200	1.7
	Hcy	40	11.8	1500[b]	560[b]	2.6

[a] ^{32}P incorporation (10 min) into PP_i from [γ-^{32}P]ATP (rate of exchange) was followed simultaneously with the ^{32}P incorporation (10 min) into the β-P of ATP (rate of interchange). Conditions were identical with those given in Table I for exchange except that 1 mM [γ-^{32}P]ATP and PP_i were used. [b] Time interval in this experiment was 20 min.

Added PP_i should affect only the exchange-mediated contribution to interchange. The various experimental approaches are described below.

Effect of Pyrophosphate Concentration on the Interchange Reaction. The effect of free PP_i on the interchange reaction was investigated with both Val- and Met-tRNA synthetases (Figure 1). In the absence of PP_i, the rates of interchange for Met and Hcy were 1800 and 4600 nmol min^{-1} mg^{-1}, respectively. In the interchange reaction catalyzed by Met-tRNA synthetase, all levels of PP_i concentration (10, 100, and 1000 μM) increased the rate of interchange dramatically with Met as substrate whereas with Hcy as substrate 10 μM PP_i did not significantly change the interchange rate and decreased the rate at 100 and 1000 μM PP_i. Similarly, for Val-tRNA synthetase, where the interchange rates in the absence of PP_i were 250, 1000, 430, and 520 nmol min^{-1} mg^{-1} for Val, Thr, Ile, and Abu, respectively, raising the PP_i concentration increased the rate of interchange with Val from about 5- to 15-fold. At 10 and 100 μM PP_i, the rates of interchange with noncognate amino acids increased slightly, at most 20%, and at 1000 μM the interchange rates decreased. With noncognate amino acids, the relative insensitivity of the interchange rates to PP_i concentration is not due to a large decrease in the K_m of PP_i compared to the K_m with cognate amino acids since with Val and Thr the K_m values of PP_i are 20 and 10 μM, respectively, in the exchange reaction. Furthermore, the lack of effect of added pyrophosphate with noncognate amino acid substrates cannot be explained by the presence of a high endogenous PP_i concentration. Such high PP_i concentrations would arise if greatly increased rates of enzyme-catalyzed ATP breakdown occurred with noncognate amino acid substrates. The rate of amino acid dependent ATP breakdown was monitored with cognate and noncognate amino acids by the appearance of [^{14}C]AMP from [^{14}C]ATP. With Val-tRNA synthetase, the rate of ATP breakdown in the presence of Val was about 10 nmol min^{-1} mg^{-1}. With the noncognate amino acids, Thr, Ile, or Abu, ATP breakdown occurred at very similar rates, 10–15 nmol min^{-1} mg^{-1}. Thus, all experiments comparing PP_i effects with cognate and noncognate amino acids, including endogenous PP_i concentrations, interchange rate variations with added PP_i, and K_m values, indicate that PP_i effects do not account for the observed differences in the ratios of direct and exchange-mediated interchange reaction rates between cognate and noncognate amino acids.

Simultaneous Measurement of Interchange and Exchange. The rates of interchange in the presence of 1 mM PP_i were calculated on the assumption that interchange resulted only from the exchange-mediated reaction, and the calculated rates were compared to experimental values. The rates of exchange and interchange were followed simultaneously by using 1 mM [γ-^{32}P]ATP and 1 mM unlabeled PP_i (Table II). The amount of ^{32}P incorporated into the β-P of ATP (representing interchange) was measured after 10 min of reaction. The amount of interchange which arises from exchange in the 10-min interval was calculated. The calculation was based on one-half (see Scheme I, reactions 2a and 2b) of the observed rate of exchange and the average specific activity of [^{32}P]PP_i during the 10-min interval. The latter equals the difference between the initial and average (over the 10-min interval) specific activities of [^{32}P]ATP. The average specific activity of [^{32}P]ATP was obtained by averaging the integrated rate equation $[[^{32}P]ATP]_t = [[^{32}P]ATP_0]e^{-kt}$ for the exchange reaction over 10 min. As shown in Table II for Val-tRNA synthetase, the ^{32}P incorporation observed exceeded the calculated ^{32}P incorporation with noncognate amino acids but not with Val, the cognate amino acid. Because of the large error in the calculated value in the case of Val, arising from the inaccuracy in determining the very fast rate of exchange with

Table III: Effect of tRNA on Interchange and Amino Acid Dependent ATP Breakdown Reactions Catalyzed by Val- and Met-tRNA Synthetases[a]

enzyme	amino acid	concn (mM)	interchange (-tRNA) (nmol min^{-1} mg^{-1})	interchange (+tRNA) (nmol min^{-1} mg^{-1})	ATP breakdown[b] (+tRNA) (nmol min^{-1} mg^{-1})
Val-tRNA synthetase	Val	2	260	1300	nd[c]
	Thr	20	880	600	630
	Ile	40	400	240	nd
	Abu	10	440	250	280
Met-tRNA synthetase	Met	2	2100	4700	nd
	Hcy	40	4600	3400	1100

[a] Conditions for interchange were identical with those for interchange in Table I except that 0.5 mM spermidine was added. The concentration of $tRNA^{Val}$ or $tRNA^{fMet}$ was 8 μM. Interchange was followed by appearance of ^{32}P in the β-P of ATP and amino acid dependent ATP breakdown by the appearance of ^{32}P in PP_i. [b] ATP breakdown not detected in the absence of tRNA. [c] nd, not detectable.

this amino acid, the observed amount of interchange is actually somewhat lower than the amount calculated from exchange. With Met-tRNA synthetase, the observed interchange with both Met and Hcy was greater than the calculated value, indicating that there is a contribution of direct interchange in both cases, but the contribution is greater with Hcy as substrate.

Role of Direct Interchange in Prevention of Misaminoacylation of tRNA

Rates of Interchange and ATP Breakdown in the Presence of tRNA. To evaluate the contribution of interchange relative to deacylation in preventing misaminoacylation, the reaction was studied in the presence of tRNA. Enzyme-catalyzed deacylation of the noncognate aminoacyl-tRNA is well established as a means of error correction after formation of the aminoacyl-tRNA (Baldwin & Berg, 1967; von der Haar & Cramer, 1976; Yamane & Hopfield, 1977). The net effect of this process is the conversion of ATP to AMP and PP_i. Interchange and ATP breakdown with cognate and noncognate amino acids were followed simultaneously in the presence and absence of tRNA (Table III). With Val-tRNA synthetase, the interchange rate with noncognate amino acids in the presence of tRNA is reduced 30–45% compared to reactions carried out in the absence of tRNA. ATP breakdown was detected only in reaction mixtures containing tRNA. With either Thr or Abu, the rate of ATP breakdown was approximately equal to the interchange rate; however, with Ile, ATP breakdown was not detected. With Met-tRNA synthetase, the interchange rate is reduced about 25% in the presence of tRNA, with Hcy as substrate, and under the latter conditions the ATP breakdown rate is only one-third of the interchange rate. The ratio of interchange to ATP breakdown rates with noncognate amino acids varies widely, ranging from about 1 (Thr, Abu) to >10 (Ile). Note that with either cognate amino acid the interchange rate increases in the presence of tRNA. Since the products formed are aminoacyl-tRNA, AMP, and PP_i and the reaction mixture contained 8 μM tRNA, 8 μM PP_i was soon generated. This newly generated PP_i increases the contribution of exchange to interchange with the cognate amino acid by increasing the rate of the reverse reaction in the interchange process (cf. Figure 1).

Rates of Interchange, Exchange, and ATP Breakdown under Physiological Conditions. Inorganic pyrophosphatase and 5 mM P_i were added to the assay system with Met-tRNA synthetase to maintain inorganic pyrophosphate concentration at its equilibrium value (Janson et al., 1979). In this way physiological conditions are mimicked more closely. Exchange, interchange, and ATP breakdown were monitored simultaneously by using both [γ-^{32}P]ATP and [^{33}P]P_i as probes. As shown in Table IV, the presence of inorganic pyrophosphatase and P_i reduces the rate of interchange about 100-fold with Met and about 18-fold with Hcy compared to controls in the absence of inorganic pyrophosphatase and P_i. The effect of inorganic pyrophosphatase is more than 5-fold lower with Hcy than with Met. Inorganic pyrophosphatase may exert an effect on that portion of the interchange that arises from the direct interchange reaction by depleting some enzyme-bound PP_i. Under conditions similar to those used in this experiment, inorganic pyrophosphatase maintains the steady-state concentration of PP_i in the range of 1 nM, according to the thermodynamic data of Alberty (1969), and in the range of 40 nM, from the experiments of Janson et al. (1979). Since the reaction mixture contains 50 nM active sites of Met-tRNA synthetase, enzyme-bound PP_i may be somewhat depleted to satisfy the pyrophosphatase equilibrium, thus decreasing the interchange rates.

Table IV: Effect of Inorganic Pyrophosphatase and tRNA on Reactions Catalyzed by Met-tRNA Synthetase[a]

additional components	exchange (nmol min^{-1} mg^{-1})	interchange (nmol min^{-1} mg^{-1})	ATP breakdown (nmol min^{-1} mg^{-1})
Met (2 mM)	–	1950	nd[b]
Met, PP_iase, [^{33}P]P_i	340	19	nd
Met, PP_iase, [^{33}P]P_i, tRNA	250	15	nd
Hcy (40 mM)	–	4600	nd
Hcy, PP_iase, [^{33}P]P_i	190	260	2100
Hcy, PP_iase, [^{33}P]P_i, tRNA	50	330	2700

[a] The exchange, interchange, and amino acid dependent ATP breakdown reactions were monitored simultaneously with two isotopic markers, ^{32}P and ^{33}P, under the conditions of Table III except that the magnesium acetate concentration was 6 mM. The concentrations of the additional components were 5 μg/mL inorganic pyrophosphatase, 5 mM [^{33}P]P_i, and 2 μM $tRNA^{fMet}$. [b] nd, not detectable.

In the presence of inorganic pyrophosphatase and P_i without tRNA, the ratio of the exchange rate to the interchange rate with Met is about 18 whereas with Hcy the ratio is about 0.7. For the cognate amino acid, Met, addition of tRNA decreases both the exchange and interchange rates slightly, but with Hcy addition of tRNA leads to a 4-fold decrease in the exchange rate while the interchange rate increases slightly. ATP breakdown was undetectable in experiments with Met as substrate (cf. Table IV). In independent experiments with [^{14}C]ATP, the increased sensitivity allowed detection of a breakdown rate with Met of <10 nmol min^{-1} mg^{-1}. When Hcy was used together with [^{14}C]ATP, a rate of breakdown of

about 250 nmol min^{-1} mg^{-1} was detected even in the absence of tRNA and inorganic pyrophosphatase. With added pyrophosphatase alone (Table IV), the breakdown rate with Hcy increased by approximately 1 order of magnitude. Inorganic pyrophosphatase and tRNA together cause the breakdown rate to increase only about 30% over the rate with inorganic pyrophosphatase alone (Table IV). In separate experiments with Val-tRNA synthetase and 40 mM Ile, 5 mM P_i, 2 μM $tRNA^{Val}$, and inorganic pyrophosphatase, the rates of interchange and ATP breakdown were 34 and 8 nmol min^{-1} mg^{-1}, respectively (not shown in Table IV).

Discussion

From the results of the experiments given above, it seems clear that with noncognate amino acids there is a direct interchange reaction distinct from the exchange-mediated interchange reaction catalyzed by Val- and Met-tRNA synthetases, whereas with cognate amino acids it appears that direct interchange is not a quantitatively significant reaction. With thio-substituted analogues of ATP where the contribution of the exchange-mediated reaction is greatly reduced relative to the rate of interchange, direct interchange is quantitatively significant even with cognate amino acids (Rossomando et al., 1979).

A comparison of the contribution of the exchange-mediated interchange to the observed interchange with a cognate (Val) and a noncognate (Thr) amino acid may be illustrated by the following example. From the Michaelis–Menten equation, $v/V_{max} = [S]/(K_m + [S])$, where v is the rate of exchange-mediated interchange, S is the PP_i concentration which is equal to enzyme concentration, K_m is the Michaelis constant of PP_i (20 μM with Val and 10 μM with Thr), and V_{max} of exchange-mediated interchange is one-half V_{max} of exchange, one may solve for v. At 2 mM Val, the observed interchange rate is 200 nmol min^{-1} mg^{-1}, and the calculated value based solely on exchange-mediated interchange, v, is 110 nmol min^{-1} mg^{-1}. On the other hand, at 10 mM Thr, the observed interchange is 775 nmol min^{-1} mg^{-1}, and the calculated v is only 14 nmol min^{-1} mg^{-1}. The large discrepancy for the noncognate Thr system must be ascribed to the contribution of the direct interchange reaction.

Just as many other mechanistic features of aminoacyl-tRNA synthetases as a class vary considerably (Kisselev & Favorova, 1974), so too with the direct interchange reaction. It is difficult to generalize the quantitative significance of the role of direct interchange in error prevention for all synthetases. The reaction is significant relative to ATP breakdown (deacylation) in the presence of tRNA (Table III), but under more physiological conditions, in which tRNA, inorganic pyrophosphatase, and P_i are present, the rate of the interchange reaction catalyzed by Met-tRNA synthetase is severalfold lower than the rate of ATP breakdown (Table IV). It may be that interchange is of major importance for those synthetases which do not have an editing mechanism operating primarily via deacylation.

Fersht & Dingwall (1979c) have shown that Val-tRNA synthetase does not have an editing mechanism for Ile involving deacylation, but rather Ile is rejected before exchange or aminoacylation takes place. The interchange reaction is one mechanism whereby this rejection could occur. Evidence for this suggestion arises from the finding that with Ile the interchange rate with Val-tRNA synthetase is more than 4 times greater than the ATP breakdown rate, in the presence of tRNA, inorganic pyrophosphatase, and P_i. Fersht & Dingwall (1979c) have argued that exchange in the presence of Ile is due to contamination of Ile with Val. The finding (cf. Table I) that the interchange rate with Ile is greater than that with Val indicates that contamination with Val cannot be invoked as an explanation for the interchange reaction. Furthermore, a contamination of Ile with Thr as the source of the interchange activity is also unlikely, considering that the observed K_m values for exchange and interchange with Ile and Thr are so similar. Moreover, with tRNA, ATP breakdown is not observed with Ile but is observed with Thr. These results strongly support the view that the reactions observed with Ile are not caused by contamination with other amino acids. Therefore, it is likely that direct interchange plays a significant role in error prevention for Ile and that both direct interchange and deacylation reactions are operative for Thr and Abu.

It has been noted (Fersht & Dingwall, 1979b) that for Met-tRNA synthetase the aminoacyl-AMP intermediate does not have to be transferred to tRNA for ATP breakdown to occur. Supporting this view is the observation that with Met-tRNA synthetase with Hcy as substrate the presence of inorganic pyrophosphatase alone (Table IV) leads to greater ATP breakdown than with tRNA (Table III). In addition, with Val-tRNA synthetase, we found that the presence of inorganic pyrophosphatase enhances ATP breakdown more than 10-fold with Thr or Abu as substrates. With these noncognate amino acids, inorganic pyrophosphatase appears to destabilize the AA-AMP by removing PP_i from the synthetases via hydrolysis of free PP_i. Even though a Val-tRNA synthetase catalyzed deacylation of aminoacyl-tRNA with its concomitant ATP breakdown does exist, the instability of the noncognate AA-AMP itself appears to contribute significantly to the editing mechanism. Jakubowski (1978), using Val-tRNA synthetase from yellow lupin seeds, has shown that enzyme-bound Thr-AMP and Abu-AMP are more unstable than Val-AMP. Although Jakubowski concluded that the instability may not be great enough to lead to an increase in fidelity, it may be, as we have found for the *E. coli* enzyme, that in the presence of inorganic pyrophosphatase the rate of ATP breakdown is greatly increased even in the absence of tRNA.

The generally accepted mechanisms (Hopfield, 1974; von der Haar & Cramer, 1976; Yamane & Hopfield, 1977; Fersht & Dingwall, 1979b) for increasing the fidelity of aminoacylation of tRNA operate at two sites for amino acid discrimination: (1) before the formation of aminoacylated tRNA, in the initial binding of substrates, and (2) after an energy-coupled irreversible step (formation of E·AA-AMP + $PP_i \rightarrow 2P_i$) which is followed by the breakdown of AA-tRNA (or AA-AMP).

The first site of discrimination (Hopfield, 1974) is based on the difference in binding constants for cognate and noncognate amino acids. This difference is of sufficient magnitude in some cases that further discrimination is unnecessary, e.g., *E. coli* cysteinyl-tRNA synthetase (Fersht & Dingwall, 1979a) and tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* (Fersht et al., 1975).

The interchange reaction described in this paper will be wholly or partially responsible for this disparity in the apparent binding constants between cognate and noncognate amino acids provided that (1) there is not a common amino acid binding site for interchange and for aminoacyl adenylate formation, but the binding of amino acid to one site prevents the binding of amino acid to the other site, and (2) the binding of amino acid at the site for the interchange reaction is favored for the noncognate amino acids relative to the cognate amino acids. On the other hand, if there is a common amino acid binding site, then the mechanism whereby the interchange

reaction may contribute to increased fidelity will occur in a step after binding but preceding the second site of discrimination, i.e., hydrolysis of aminoacylated products. If the reaction of ATP and amino acid leads to a common intermediate for the interchange and aminoacylation reactions before dissociation of PP_i from the enzyme, the common intermediate will be partitioned between the two reactions. Consequently, the amount of enzyme available for the aminoacylation pathway will decrease. Since the alternate pathway (interchange) is quantitatively much more significant with noncognate amino acids as substrates, the overall rate of aminoacylation of tRNA by noncognate amino acids as substrates, relative to cognate amino acids, will decrease. For cognate amino acids, the rate of interchange relative to the rate of formation of aminoacyl adenylate and free PP_i (first partial reaction in aminoacylation) is much lower (or nonexistent) than for the noncognate amino acids so that the overall rate of aminoacylation is hardly affected. Thus, discrimination results because only noncognate amino acids divert the enzyme to a nonproductive pathway, thus decreasing their rate of aminoacylation. Evidence for the direct interchange reaction should be sought for other synthetases, particularly those in which the second site of discrimination (deacylation) is unimportant, e.g, cysteinyl-tRNA synthetase (Fersht & Dingwall, 1979a) and tyrosyl-tRNA synthetase (von der Haar & Cramer, 1976).

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Arachidonic Acid Releasing Activity in Platelet Membranes: Effects of Sulfhydryl-Modifying Reagents†

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ABSTRACT: The effects of sulfhydryl-modifying agents on arachidonic acid releasing activity in isolated platelet membranes were studied. Release of arachidonic acid was inhibitable by 5,5′-dithiobis(2-nitrobenzoic acid) and *N*-ethylmaleimide but not by diazenedicarboxylic acid bis(dimethylamide) (diamide). A total of 51.4 nmol of sulfhydryls/mg of membrane protein reacted with 5,5′-dithiobis(2-nitrobenzoic acid) as four kinetically distinguishable classes, forming, in addition to mixed disulfides, inter- and/or intraprotein disulfides. While diamide cross-linking of 85% of the membrane sulfhydryls caused no inhibition, subsequent incubations with either *N*-ethylmaleimide or 5,5′-dithiobis(2-nitrobenzoic acid) inhibited the releasing activity to the same extent as in the membranes which had not been previously cross-linked. Therefore, modification of a few "essential" sulfhydryls was responsible for the rapid inhibition of arachidonic acid releasing activity. Membranes isolated from 5,5′-dithiobis(2-nitrobenzoic acid)-treated intact platelets exhibited less inhibition than membranes similarly treated following isolation. 5,5′-Dithiobis(2-nitrobenzoic acid) (4 mM) inhibited the former by 20% and the latter by 92%. Since no such difference in inhibition was observed with *N*-ethylmaleimide, it is possible that the membrane vesicles are either "inside out" or open to 5,5′-dithiobis(2-nitrobenzoic acid) which does not penetrate membranes [Smith, R. P. P., & Ellman, G. L. (1973) *J. Membr. Biol. 12*, 177–188]. The results are consistent with a model whereby essential sulfhydryls may be located on the inner surface of the platelet membrane. This is also supported by studies where the same 5,5′-dithiobis(2-nitrobenzoic acid) concentration inhibited oxygen consumption of thrombin- or collagen-stimulated platelets by 15–28%. These results suggest that the same enzyme(s) are involved in both arachidonic acid releasing activity in platelet membranes and in arachidonic acid mobilization in stimulated intact platelets.

Mobilization of arachidonic acid from the 2 position of platelet phospholipids is the first step in thromboxane and hydroxy acid synthesis (Marcus, 1978; Lands, 1979). Whereas there is a broader understanding of the thromboxane and hydroxy acid pathways, mechanisms involved in release of arachidonic acid are less well defined. Since thromboxane synthesis is only one of several biological responses of stimulated platelets (Schafer & Handin, 1979; Mustard & Packham, 1977), an understanding of arachidonate mobilization through the study of functional parameters has been difficult (Vanderhoek & Feinstein, 1978; Vallee et al., 1979).

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0006-2960/81/0420-0391$01.00/0