β- and γ-Thio Analogues of Adenosine Triphosphate as Probes of the *Escherichia coli* Valyl Transfer Ribonucleic Acid Synthetase Reaction Pathway. A Novel Stereospecific Interchange of Adenosine 5′-*O*-(2-Thiotriphosphate) to Adenosine 5′-*O*-(3-Thiotriphosphate)†

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ABSTRACT: ATPγS and the diastereomers of ATPβS were compared to ATP as substrates for the pyrophosphate (PP_i) exchange and aminoacylation reactions catalyzed by the valyl-tRNA synthetase from *Escherichia coli*. The rates with ATPγS were similar to those with ATP, whereas the rates with ATPβS were reduced at least by 1–2 orders of magnitude. While the overall aminoacylation reaction was stereoselective for the B diastereomer by a factor of about 3.5, the A diastereomer was the preferred substrate for the exchange reaction by a factor of about 90. The anomalous difference in stereospecificity is explicable in terms of a novel reaction catalyzed by the enzyme, the formation of ATPγS from ATPβS in the absence of PP_i, designated the interchange reaction. This reaction is characterized by a high stereospecificity (>500) for the A diastereomer, opposite to the stereoselectivity of aminoacylation. Further, the equilibrium for the interchange reaction lies far toward ATPγS. The rate of aminoacylation from ATPβSA is similar to the rate obtained for formation of ATPγS from ATPβSA, suggesting that the aminoacylation observed with this analogue resulted from the intermediate formation, by interchange, of ATPγS which then serves as the substrate for aminoacylation. Comparable rates of interchange and of exchange of ATPβSA with PP_i imply that thiopyrophosphate (SPP_i) is not released during the interchange reaction and that the process of interchange occurs while the SPP_i remains bound to the enzyme. Possible mechanisms as well as a possible physiological role for the interchange reaction are discussed.

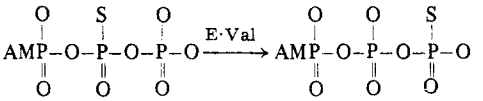
Current evidence suggests that most aminoacyl-tRNA synthetases catalyze the production of aminoacyl-tRNA by the following two-step reaction mechanism (Loftfield, 1972):

$$E + AA + ATP \overset{\text{fast}}{\rightleftharpoons} E{\cdot}AA{-}AMP + PP_i \quad (1)$$

$$E{\cdot}AA{-}AMP + tRNA \overset{\text{slow}}{\rightleftharpoons} AA{-}tRNA + AMP + E \quad (2)$$

Analogues of ATP in which a nonbridge oxygen on either α-, β-, or γ-P is replaced by sulfur have been used as probes of these reactions (Pimmer et al., 1976; von der Haar et al., 1977). For both ATPαS and ATPβS,[1] a pair of diastereomers exists which may be used to determine the stereospecificity of enzymatic reactions. However, an investigation of the aminoacylation reaction catalyzed by phenylalanyl-tRNA synthetase from *Escherichia coli* showed, rather unexpectedly, that the maximum velocities of aminoacylation obtained with all five thiophosphate analogues of ATP (ATPγS, ATPβS, A and B, and ATPαS, A and B) as substrates were reduced relative to ATP by very similar factors ranging from 3 to 10 (Pimmer et al., 1976). Neither the chirality of the substrate nor the position of the sulfur affected the rate of aminoacylation appreciably.

In the present study, ATPγS and the ATPβS diastereomers were compared to ATP as substrates for the reactions catalyzed by valyl-tRNA synthetase from *E. coli*. On the basis of the sequence formulated in eq 1 and 2, the second step should be common to all four substrates. Therefore, any differences in rate or stereospecificity encountered in the overall aminoacylation reaction should be ascribable to differences in these parameters in the first step. These two steps were differentiated by comparing the first partial reaction, an exchange reaction with PP_i in the absence of tRNA, with the overall aminoacylation. In the exchange reaction with ATP thio analogues, total nucleotide is conserved but added PP_i exchanges for SPP_i to form ATP from ATPS. A third reaction was observed and characterized, an interchange reaction, in which ATPβSA is converted to ATPγS in the absence of added PP_i, i.e.



(3)

The contribution of this interchange reaction to the rate and stereospecificity of both the exchange and aminoacylation reactions of ATPβS is analyzed.

Materials and Methods

Materials. *E. coli* (K-12) was obtained from Grain Processing Corp. Agarose–hexane–AMP (type 3), ATPγS, and ADPβS were purchased from P-L Biochemicals, Inc. ATP, unfractionated tRNA (*E. coli*), phosphoenolpyruvate, acetyl phosphate, and acetate and adenylate kinases were purchased from Sigma Chemical Co., and spermidine was from Calbiochem. [^{32}P]PP_i was purchased from New England Nuclear. [^{3}H]AMP (15 Ci/mmol) and [^{14}C]-L-valine (50 mCi/mmol) were obtained from Schwarz/Mann. Hexokinase (P-II) was a product of Worthington Biochemical Co., inorganic pyro-

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[1] Abbreviations used: BSA, bovine serum albumin; TEA, triethylamine; ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); ATPβS, adenosine 5′-*O*-(2-thiotriphosphate); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PP_i, pyrophosphate; SPP_i, thiopyrophosphate.

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phosphatase was a gift from B. Cooperman of the University of Pennsylvania, and pyruvate kinase was from D. Ash of the University of Pennsylvania. Triethylamine (Eastman) was distilled before use. PEI–cellulose (F-5504) plastic sheets were purchased from Merck, valinol was from Aldrich Chemical Co., and Instabray scintillation solution was from Yorktown Research. Diethylaminoethylcellulose (DE-52) was from Whatman, Ltd. All other chemicals were reagent grade.

Preparation of Enzymes. The valyl-tRNA synthetase was purified by modification of the method previously described (Yaniv & Gros, 1969). The calcium phosphate gel fractionation step was omitted and additional purification involved chromatography on an AMP–Sepharose column as described for the methionyl enzyme (Fayat et al., 1977) but in the presence of 8 mM $MgCl_2$ and 5 mM valinol and elution with 20 mM L-valine. Methionyl-tRNA synthetase from *E. coli* was prepared according to Bruton et al. (1975).

Preparation of ^{3}H-Labeled and Unlabeled Thiophosphate Analogues. [^{3}H]ADPβS and [^{3}H]ATPγS were prepared enzymatically with adenylate kinase in a 10-mL volume containing 30 mM Tris-HCl, pH 8.1, 5 mM $MgCl_2$, 5 mM dithioerythritol, 2 mM ATPγS, and 1 mCi of [^{3}H]AMP (6 μM). After addition of adenylate kinase, the reaction proceeded for about 2 h at room temperature. At the end of the incubation, 5 μmol of unlabeled ADPβS was added and the reaction mixture was applied to a DE-52 column (1.5 × 15 cm). The products, ATP, ADP, AMP, ATPγS, and ADPβS, eluted with a 3-L gradient of TEA bicarbonate buffer, pH 7.6 (0.05-0.5 M), were well resolved from each other. The fractions containing [^{3}H]ATPγS and those containing [^{3}H]-ADPβS were pooled separately, evaporated to dryness, dissolved in water, and stored frozen. [^{3}H]ADPβS and [^{3}H]-ATPγS thus obtained had specific activities of about 5×10^7 and 5×10^8 cpm/μmol, respectively.

ATPβSA and -B, both ^{3}H-labeled and unlabeled, were prepared enzymatically from ^{3}H-labeled and unlabeled ADPβS, respectively, as described previously (Eckstein & Goody, 1976; Jaffe & Cohn, 1979). The [^{3}H]ATPβSA and -B obtained had specific activities of about 10^7 cpm/μmol. A 20–30% yield of [^{3}H]ATPβSA and -B from [^{3}H]AMP was usually obtained.

Aminoacylation Reaction Assay. The aminoacylation reaction was monitored by the production of [^{14}C]valyl-tRNA under the following conditions: 40 mM Tris-HCl, pH 8.1, 2 mM spermidine, 100 μM [^{14}C]valine (4×10^7 cpm/μmol), 6 mM 2-mercaptoethanol, 0.2 mg/mL BSA, 0.2 mg of unfractionated tRNA, 0.075–5 mM ATP or thio analogue, magnesium acetate equimolar to ATP or ATPS, for ATPβS additional 6 and 12 mM magnesium acetate for the A and B diastereomers, respectively, and 0.02–0.2 μg of enzyme, in a final volume of 100 μL. The concentration of excess magnesium used was that magnesium concentration which yielded maximum velocity at 2 mM adenine nucleotide. The reaction mixture, after addition of enzyme, was incubated at 30 °C for 10 min. The reaction was terminated by addition of trichloroacetic acid (final concentration 5%). The radioactivity in the product was determined as described previously (Posorske et al., 1979).

Exchange Reaction Assay. The exchange was followed by the production of [^{32}P]ATP from [^{32}P]PP_i under the following conditions: 40 mM potassium Hepes, pH 8, 2 mM valine, 6 mM 2-mercaptoethanol, 0.2 mg/mL BSA, 1–2 mM [^{32}P]PP_i (1×10^6 cpm/μmol), 0.1–2 mM ATPS, total magnesium acetate to a 5 mM excess over an equimolar concentration of ATPS, and 0.1–15 μg of enzyme, in a final volume of 50 μL.

Table I: Comparison of Rates for ATP and Thio Analogues in the Aminoacylation Reaction of *E. coli* Val-tRNA Synthetase

	aminoacylation[a]	
substrate	V_{max} [nmol/(min mg)]	K_m (mM)
ATP	1400	0.12
ATPγS	800	0.2
ATPβSA	46	0.3
ATPβSB	160	0.3

[a] Maximum velocities and Michaelis constants were determined at 30 °C under the following conditions: 40 mM Tris-HCl, pH 8, 100 μM valine, 6 mM 2-mercaptoethanol, 2 mM spermidine, 0.2 mg/mL BSA, and 2 mg/mL unfractionated tRNA. Mg^{2+} and ATP and ATP analogues were varied as noted under Materials and Methods.

The reaction was terminated by the addition of 30 μg of yeast inorganic pyrophosphatase, an amount sufficient to hydrolyze all of the PP_i present in about 1 min. After 10 min at room temperature, 1 μL of a 20 mM solution of ATP was added to the reaction mixture as a carrier. Two 5-μL aliquots of the mixture were spotted on a PEI–cellulose sheet which was developed for 30 min in 0.38 M KH_2PO_4, pH 3.5. The position of the ATP was determined under ultraviolet light. The ATP spot was cut out, and the amount of radioactivity was determined by liquid scintillation counting directly in 5 mL of Instabray. Rates were proportional to enzyme concentration and linear with time over the period of incubation.

Interchange Reaction Assay. [^{3}H]ATPβSA or -B (0.1 μmol) containing about 60 000 cpm was dissolved in 50 μL of a solution containing 40 mM potassium Hepes, pH 8, 5 mM magnesium acetate, 2 mM valine, 6 mM 2-mercaptoethanol, and 5 μg of BSA. Reaction was initiated with valyl-tRNA synthetase (5–20 μg) and proceeded at 30 °C. At intervals, two 5-μL aliquots were removed; the radioactive compounds were counted after chromatographic separation as described above, except that 0.75 M KH_2PO_4, pH 3.5, was used as the developing solvent.

The interchange reaction was also followed by ^{31}P NMR. A typical reaction mixture contained 20 mM Hepes, pH 8, 10 mM magnesium acetate, 2 mM valine, 1 mM EDTA, 6.2 mM ATPβSA, 470 μg of valyl-tRNA synthetase, and 20% D_2O. ^{31}P NMR spectra were recorded on 500-μL samples in a 10-mm microcell at 145.7 MHz on a Bruker WH360 spectrometer, with broad-band proton decoupling.

Results

Aminoacylation. The apparent K_m and the V_{max} values for ATP, ATPγS, and ATPβSA and -B in the aminoacylation reaction listed in Table I were determined from double-reciprocal plots ($1/v$ vs. $1/[S]$). Substitution with sulfur on the γ-P reduces the rate of aminoacylation only 40%, but substitution on the β-P reduces the rate more severely and differentially for the two diastereomers, about 30-fold with MgATPβSA and about 9-fold with MgATPβSB. The various apparent K_m values differ significantly less than the corresponding V_{max} values, although the order of the two parameters is the same: i.e., V_{max}, ATP > ATPγS > ATPβS; K_m, ATP < ATPγS < ATPβS. In contrast to ATP and ATPγS, both diastereomers of ATPβS cause pronounced substrate inhibition of the aminoacylation reaction, effective at concentrations above 0.2 mM for B and above 1 mM for A, as shown in Figure 1.

Interchange of β- and γ-P Groups of ATPβS and ATPγS. When the [^{3}H]ATPβSA analogue was incubated with valine and enzyme in the absence of PP_i, it was found, after analysis of the reaction mixture by thin-layer chromatography, that

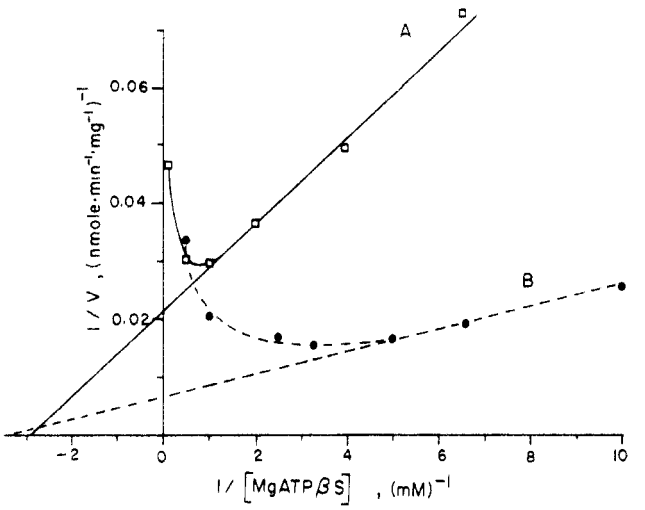

FIGURE 1: Comparison of kinetic parameters and substrate inhibition for ATPβSA and ATPβSB in the formation of valyl-tRNA. (A) MgATPβSA, 0.15–5.0 mM; (B) MgATPβSB, 0.1–2.0 mM.

Table II: Comparison of Rates for ATP Thio Analogues in the Interchange Reaction of *E. coli* Val-tRNA Synthetase

substrate	interchange[a] V [nmol/(min mg)]
ATPγS	<0.1
ATPβSA	50
ATPβSB	<0.1

[a] Velocities were determined under the following conditions at 30 °C: 40 mM Hepes, pH 8, 2 mM valine, 6 mM 2-mercaptoethanol, 5 mM magnesium acetate, 1 mM ATP analogue, and 0.2 mg/mL BSA.

ATPβSA was quantitatively converted to a compound which migrated with authentic ATPγS. The initial rate of the conversion is 50 nmol/[min (mg of enzyme)] (Table II). After an incubation of 12–16 h, no ATPβSA remained and all the radioactivity was recovered as ATPγS.

The reaction was also followed by ^{31}P NMR spectroscopy. Figure 2 shows a representative spectrum of ATPβSA and two of the spectra taken during the course of the incubation. Figure 2A shows the ^{31}P spectrum of the initial ATPβSA. The three new peaks which appear after 50-min incubation (Figure 2B) correspond to those of ATPγS as assigned from known chemical shifts (Jaffe & Cohn, 1978). This spectrum confirms unequivocally the conclusion derived from the chromatographic analysis that ATPβSA is being converted to ATPγS during the course of the incubation with the valyl-tRNA synthetase. After 240 min, only ATPγS is observed as shown in Figure 2C; ATPβSA has been completely converted to the γS isomer. From measurements of the ATPβSA peak heights in the ^{31}P NMR spectra as a function of time, the rate for this interchange reaction under the conditions used is ~50 nmol/(min mg).

Similar experiments were carried out with [^{3}H]ATPβSB as the substrate. After incubation for as long as 24 h no evidence for formation of [^{3}H]ATPγS was observed. Based on estimates of our limits of detection, the maximum rate of [^{3}H]ATPγS formation would be 0.1 nmol/(min mg) (Table II).

In other experiments [^{3}H]ATPγS was incubated with valyl-tRNA synthetase under interchange reaction conditions, and, again, after incubations for as long as 24 h no evidence for formation of [^{3}H]ATPβS was observed (Table II). This finding, taken together with the observation that ATPβSA is completely converted to ATPγS, indicates that ATPγS is

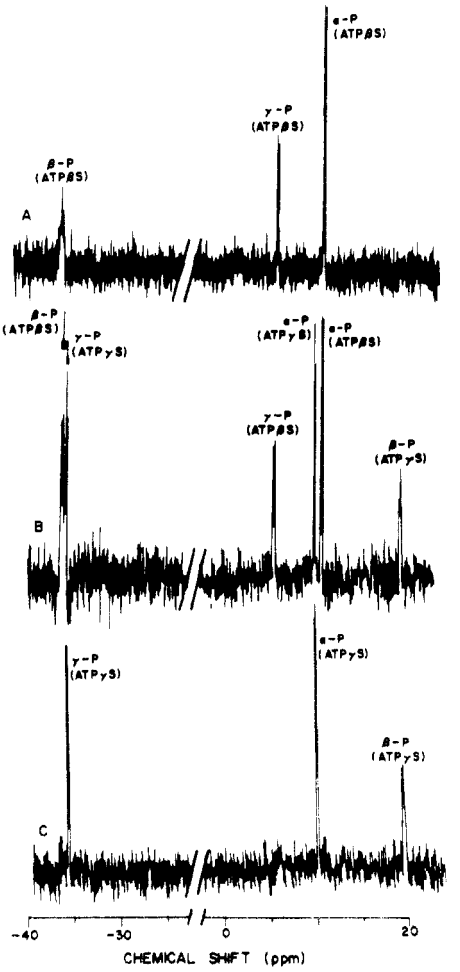

FIGURE 2: ^{31}P NMR spectra at 145.7 MHz of the interchange reaction, conversion of ATPβSA to ATPγS. The chemical shifts are relative to 85% H_3PO_4 as the external reference. (A) Initial spectrum of ATPβSA, (B) after 50 min, and (C) at equilibrium, after 240 min. NMR parameters: spectral width, 16.5 kHz; repetition time, 0.62 s; line broadening, 2 Hz; 1660 scans.

thermodynamically more stable than ATPβS.

A comparison of the data presented in Tables I and II also shows that the interchange reaction is stereospecific for the A isomer, $V_B/V_A < 0.002$; the preference for the A isomer is reversed in the aminoacylation reaction where $V_B/V_A = 3.5$. These data indicate that interchange and aminoacylation follow separate reaction pathways. The remote possibility that the binding of tRNA to the enzyme could reverse the stereospecific constraints of the enzyme's nucleotide binding site was eliminated by investigating the effect of $tRNA^{Val}$ on the interchange reaction. Although the rate of the interchange reaction was slightly reduced at equimolar levels of tRNA and enzyme, the stereospecificity was unchanged. Further evidence that the interchange reaction is distinct from the aminoacylation reaction arises from the finding that although K_m values are almost equal for the two diastereomers in the aminoacylation reaction (see Table I), an analogous relationship does not appear to hold in the interchange reaction. In the latter reaction there is no significant decrease in the rate with 2 mM ATPβSA as the substrate in the presence of 0.02 to 2 mM ATPβSB.

A requirement for valine was demonstrated in the interchange reaction. Valinol, which has a K_i about equal to the K_m of valine (Loftfield, 1972), could not substitute for valine when tested over a concentration range of 2–8 mM; no reaction was observed even after a 20-h incubation. Isoleucine, which

Table III: Comparison of Rates for ATP and Thio Analogues in the Exchange Reaction of *E. coli* Val-tRNA Synthetase

substrate	exchange[a] V [nmol/(min mg)]
ATP	10 800
ATPγS	3 100
ATPβSA	71
ATPβSB	0.8

[a] Velocities for exchange were determined under identical conditions as those for interchange except that 1 mM PP_i was added.

can substitute for valine in the ATP–PP_i exchange reaction catalyzed by the valyl enzyme (Yaniv & Gros, 1969), also substitutes for valine in the interchange reaction. Thus, we observed similar rates of interchange with either 2 mM valine or 4 mM isoleucine.

Experiments carried out with methionyl-tRNA synthetase from *E. coli* under conditions similar to those described for the valyl enzyme exhibit a similar pattern. With the methionyl enzyme, ATPβSA but not ATPβSB is converted to ATPγS. Also, no reaction was observed in the reverse direction, starting from ATPγS. Whether catalysis of the interchange reaction is a common feature of all aminoacyl-tRNA synthetases and perhaps other nucleotidyl transfer reactions remains to be established.

Exchange Rate of ATP Thio Analogues. Several experiments were designed to determine whether the interchange reaction is occurring by reversal of partial reaction 1 or is distinct from the usual ATP–PP_i exchange reaction and is occurring on the enzyme before dissociation of SPP_i from the enzyme. The results of exchange studies with PP_i and the thio analogues of ATP as the substrates are shown in Table III. The rate of exchange of ATPγS is only 3–4-fold less than that observed with ATP and about 150- and 13 500-fold greater than that observed with ATPβSA and -B, respectively. The stereospecificity for the exchange reaction is qualitatively similar to that observed in the interchange reaction, but the V_B/V_A ratio is increased at least fivefold. No change in stereoselectivity for ATPβS in the exchange reaction was observed when the ATPβS concentration was varied from 0.1 to 1 mM.

The rates of formation of [^{3}H]ATP from [^{3}H]ATPβSA at various concentrations of PP_i were investigated with ATPβSA present at 1 mM. As expected from results of interchange experiments in the absence of PP_i, only ATPγS is formed. With the addition of 0.1 mM PP_i, there is a 25% decrease in rate of ATPγS formation, and ATP is also produced. At 1 mM PP_i, a concentration equal to that of ATPβSA, the observed rate of ATPγS formation is 30% of the rate observed in the absence of PP_i, showing that the interchange reaction can occur even in the presence of PP_i equimolar with nucleotide. Furthermore, with ATPβSA as substrate the approximate equality of the rate of ATP formation in the presence of 1 mM PP_i (cf. Table III) and of the rate of interchange in the absence of PP_i (cf. Table II) suggests that interchange is not occurring via an exchange mechanism.

Discussion

Dramatic differences in the rate of aminoacylation catalyzed by *E. coli* valyl-tRNA synthetase do occur among ATP and its β- and γ-thio analogues as the substrates, despite the fact that the second step, reaction 2, is presumably common to these substrates. Thus, if the two-step sequence is applicable to the thio analogues, step 1 must become partially, or wholly, rate limiting. The position of the sulfur affects the rate of aminoacylation catalyzed by the valyl enzyme to a greater degree than for the rates reported for the phenylalanyl enzyme; with the valyl enzyme, the rates fall in the order ATP > ATPγS > ATPβS.[2] The pattern agrees with the generalization (Nogc et al., 1979) that substitution of an oxygen of ATP by sulfur reduces the rate of reaction progressively as the position of the sulfur approaches the site of bond cleavage.[2] The results of experiments in which PP_i exchanges for SPP_i to form ATP from ATPβS follow the same pattern. The rates relative to ATP are as follows: ATPγS, 0.29; ATPβSA, 0.0066; ATPβSB, 0.00074.

Since the stereospecificity of the exchange reaction is the reverse of the aminoacylation reaction with ATPβS, eq 1 and 2 cannot represent the pathway of aminoacylation for ATPβSA or -B or both. The origin of this reversal of stereospecificity is the interchange reaction which becomes of primary importance in the reaction sequence of ATPβSA. The interchange reaction is highly stereospecific; the preference for ATPβSA to -B in the Mg^{2+}-activated reaction is at least 500-fold. Since the product of this interchange reaction is ATPγS, the utilization of ATPβSA in both the exchange and aminoacylation reactions may be due to the initial formation of ATPγS and the rapid reaction of the latter in subsequent reactions. The similarity among the rates of interchange, exchange, and aminoacylation with ATPβSA as the substrate lends credence to this interpretation. Any contribution of the interchange reaction, i.e., the conversion of ATPβSA to ATPγS, affects the stereospecificity of the exchange reaction more than that of aminoacylation because the ratio of the exchange rate to aminoacylation rate is ~4 for ATPγS, but for ATPβSB the ratio is 0.005 (cf. Tables I and III), and consequently the measured stereospecificity for B in aminoacylation is lowered but for exchange it is reversed. On the basis of these findings, it is clear that the apparent stereospecificity observed with "pyrophosphorylases" with ATPβS diastereomers as substrates may be misleading if, as in the case of valyl-tRNA synthetase, an interchange reaction of opposite stereospecificity from the overall reaction were to be interposed in the reaction sequence of one of the diastereomers.

The reaction pathway for ATP represented by steps 1 and 2 is inapplicable for the thio analogues. Since with ATPβSB, unlike ATP, the rate of aminoacylation greatly exceeds the rate of exchange with PP_i, then SPP_i must be released at an appreciable rate only after tRNA has interacted with the enzyme·valyladenylate complex and two separable steps cannot exist. With ATPβSA, the interchange reaction to form ATPγS makes a straightforward analysis difficult.

The pattern of the reaction rates for ATPβS is intermediate between that for ATP with the valyl enzyme and the pattern for ATP with arginyl-, glutamyl-, and glutaminyl-tRNA synthetases. For the latter group of enzymes, no evidence for exchange between ATP and PP_i can be obtained in the absence of tRNA (Mehler & Mitra, 1967; Ravel et al., 1965). The difference between the reaction pathways of ATPβSA and -B must be ascribed to (1) different modes of binding of substrates to the enzyme at the same binding site or (2) the ability of ATPβSA to bind preferentially to a second site. The observation that ATPβSB, the preferred diastereomer for aminoacylation, at concentrations about its K_m, does not inhibit the interchange reaction of ATPβSA strongly favors the second possibility.

The interchange reaction with ATPβSA occurs on the enzyme before dissociation of the thiopyrophosphate group from the enzyme. The requirement for valine in the interchange

[2] The rate of aminoacylation catalyzed by the valyl enzyme for the ATPαS diastereomers is about threefold lower than the rate for ATPβS (L. T. Smith and M. Cohn, unpublished experiments).

reaction, as indicated by the observation that it is not replaceable by valinol, indicates that the carboxyl group participates either because valyladenylate is an intermediate in the interchange reaction or because it induces an essential conformational change in the enzyme upon binding.

The interchange might occur by a one-step mechanism, whereby the α–β bond cleavage and α–γ bond formation would occur simultaneously. As an alternative, a two-step sequence in which either a valyladenylate– or pyrophosphoryl–enzyme intermediate (Cooper & Kornberg, 1969) would be formed might be considered.

The finding that the valyl-tRNA synthetase can catalyze the readily observed β- to γ-P interchange of ATPβS raises the question of the physiological significance of this reaction, if any, with ATP as the substrate. One possible function is suggested by our observation that isoleucine can readily substitute for valine in the interchange reaction. This finding suggests that the interchange reaction may function as a mechanism for editing, i.e., a mechanism by which the "wrong" amino acid could interact with the synthetase but would not result in aminoacyl-tRNA formation. Although other mechanisms for editing have been proposed and investigated (Fersht & Dingwall, 1979; von der Haar & Cramer, 1976; Yamane & Hopfield, 1977), the interchange reaction reported in this paper, which would cycle the wrong amino acid in a nonproductive reaction resulting in no loss of ATP, may contribute yet an additional mechanism for ensuring the fidelity of aminoacylation. This possibility is presently being investigated.

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Affinity Labeling of a Reactive Sulfhydryl Residue at the Peptidyl Transferase P Site in *Drosophila* Ribosomes†

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ABSTRACT: An affinity label has been prepared that is specific for the P site of an eucaryotic peptidyl transferase, that of *Drosophila melanogaster*. It has the sequence C-A-C-C-A-(Ac[^{3}H]Leu) with a mercury atom added at the C-5 position of all three cytosine residues (referred to as the mercurated fragment). This label is an analogue of the 3′ terminus of *N*-acetylleucyl-tRNA. The mercurated fragment binds specifically to the P site of peptidyl transferase. It participates fully in peptide bond formation as judged by its ability to transfer *N*-acetylleucine to puromycin with at least the same efficiency as a nonmercurated fragment. Once bound to the P site, the mercurated fragment reacts covalently with a ribosomal protein(s). This affinity-labeling process can be effectively competed by nonmercurated fragment, which indicates a site-specific reaction. The covalent attachment of the affinity label to a ribosomal protein(s) occurs through the formation of a mercury–sulfur bond, as judged by its lability in the presence of thiol reducing agents. The major ribosomal protein labeled at the P site of *D. melanogaster* was found to be a small, basic protein. The electrophoretic behavior of this protein parallels that of major P site proteins found in *Escherichia coli* ribosomes and in other eucaryotes. These results suggest conservation of some of the overall properties of the P site proteins from these organisms.

Affinity-labeling techniques have been instrumental in the investigation of the 70S ribosome of *Escherichia coli*. Various structural components have been correlated with particular ribosomal functions using these specifically designed probes. Numerous affinity-labeling experiments, in addition to data from cross-linking, immune electron microscopy, and chemical modification experiments, have contributed to an understanding of the three-dimensional structure of the bacterial ribosome and of the molecular events which occur during protein synthesis.

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