fragments hybridizing with the cDNA probe extended to more than 11 kb with 5′ and 3′ flanking regions of considerable length, which showed no hybridization signal with the cDNA. Therefore, clones pgP-450pb-1 and -8 may cover the whole sequence of the cytochrome P-450 gene.

Acknowledgments

We thank Drs. T. D. Sargent, R. B. Wallace, and J. Bonner for the rat genomic library of partial *Eco*RI digests.

Registry No. Cytochrome P-450, 9035-51-2; phenobarbital, 50-06-6.

References

Albert, S., Johnson, R. M., & Wagshal, R. R. (1953) *Science (Washington, D.C.) 117*, 551–553.

Benton, W. D., & Davis, R. W. (1977) *Science (Washington, D.C.) 196*, 180–182.

Blattner, E. R., Williams, B. G., Bled, A. E., Denniston-Thompson, K., Feber, H. E., Eurlong, L. A., & Smithies, O. (1977) *Science (Washington, D.C.) 196*, 161–169.

Botelho, L. H., Ryan, D. E., & Levin, W. (1979) *J. Biol. Chem. 254*, 5635–5640.

Fujii-Kuriyama, Y., Taniguchi, T., Mizukami, Y., Sakai, M., Tashiro, Y., & Muramatsu, M. (1981) *J. Biochem. (Tokyo) 89*, 1869–1879.

Fujii-Kuriyama, Y., Mizukami, Y., Kawajiri, K., Sogawa, K., & Muramatsu, M. (1982a) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2793–2797.

Fujii-Kuriyama, Y., Mizukami, Y., Taniguchi, T., & Muramatsu, M. (1982b) *Microsomes, Drug Oxidations and Drug Toxicity* (Sato, R., & Kato, R., Eds.) p 589–596, Japan Scientific Society Press, Tokyo, and Wiley-Interscience, New York.

Gross-Bellard, M., Ouduct, P., & Chambon, P. (1973) *Eur. J. Biochem. 36*, 32–38.

Grunstein, M., & Hogness, D. S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3916–3965.

Maniatis, T., Jeffrey, A., & Kleid, D. G. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1184–1189.

Sakai, M., Fujii-Kuriyama, Y., & Muramatsu, M. (1978) *Biochemistry 17*, 5510–5515.

Sato, R., & Omura, T., Eds. (1978) *Cytochrome P-450*, Kodansha, Tokyo, and Academic Press, New York and London.

Smith, H. O., & Birnstiel, M. L. (1976) *Nucleic Acids Res. 3*, 2387–2399.

Southern, E. (1975) *J. Mol. Biol. 98*, 503–517.

Tabak, H. F., & Flavell, R. A. (1978) *Nucleic Acids Res. 5*, 2321–2332.

A Stereochemical and Positional Isotope Exchange Study of the Mechanism of Activation of Isoleucine by Isoleucyl-tRNA Synthetase from *Escherichia coli*†

Gordon Lowe,* Brian S. Sproat, Gaynor Tansley, and Paul M. Cullis

ABSTRACT: Isoleucyl-tRNA synthetase from *Escherichia coli* catalyzes the activation of [$^{18}O_2$]isoleucine by adenosine 5′-[(*R*)-α-^{17}O]triphosphate with inversion of configuration at phosphorus. Moreover, isoleucyl-tRNA synthetase does not catalyze positional isotope exchange in adenosine 5′-[β-$^{18}O_2$]triphosphate in the absence of isoleucine or in the presence of the competitive inhibitor isoleucinol, which effectively eliminates the possibility of either adenylyl–enzyme or adenosine metaphosphate intermediates being involved. Together, these observations require that isoleucyl-tRNA synthetase catalyzes the activation of isoleucine by associative "in line" nucleotidyl transfer. The synthesis of adenosine 5′-[(*R*)-α-^{17}O]diphosphate and its conversion to adenosine 5′-[(*R*)-α-^{17}O]triphosphate is described and an explanation provided for the reported differences between the treatment of adenosine 5′-[(*S*)-α-thiodiphosphate] with cyanogen bromide and bromine in [^{18}O]water.

The aminoacyl-tRNA synthetases are a family of enzymes that activate amino acids with ATP and then transfer them to their cognate tRNA (Söll & Schimmel, 1974; Schimmel & Söll, 1979). The high fidelity observed in the translation of genetic information in protein biosynthesis is made possible by the ability of the aminoacyl-tRNA synthetases to selectively bind and couple their respective amino acid and tRNA and to hydrolyze ("edit") mischarged tRNA (Fersht, 1981). The activation of an amino acid by ATP to form an aminoacyl adenylate can be studied in the absence of its cognate tRNA with most aminoacyl-tRNA synthetases, and it is now accepted that all aminoacyl-tRNA synthetases adopt this two-step process (Kim et al., 1977):

$$H_3N^+\cdot CHR\cdot CO_2^- + MgATP \rightleftarrows H_3N^+\cdot CHR\cdot CO\cdot AMP + MgPP_i$$

$$H_3N^+\cdot CHR\cdot CO\cdot AMP + tRNA \rightleftarrows H_3N^+\cdot CHR\cdot CO\cdot tRNA + AMP$$

The structural diversity of the aminoacyl-tRNA synthetases is rather surprising. They occur as monomers, dimers, and tetramers and manifest considerable differences in subunit molecular weight (Kiselev & Favorova, 1974). There is however evidence to suggest that the larger subunits may have arisen by gene duplication and fusion (Kalousek & Konigsberg, 1976; Waterson & Konigsberg, 1974; Koch et al., 1974; Kula, 1973). The question we wish to consider concerns the

† From the Dyson Perrins Laboratory, Oxford University, Oxford OX1 3QY, U.K. (G.L., B.S.S., and G.T.), and the Department of Chemistry, Leicester University, Leicester LE1 7RH, U.K. (P.M.C.). *Received August 26, 1982.* This work was supported by a grant from the Science and Engineering Research Council and is a contribution from the Oxford Enzyme Group.

0006-2960/83/0422-1229$01.50/0

mechanism of activation of amino acids by aminoacyl-tRNA synthetases and whether mechanistic differences are associated with structural diversity. We have selected for initial study isoleucyl-tRNA synthetase from *Escherichia coli*, a monomer of molecular weight about 110000 (Baldwin & Berg, 1966; Arndt & Berg, 1970; Berthelot & Yaniv, 1970; Durekovic et al., 1973).

Amino acid activation involves nucleophilic substitution at P_α of MgATP with displacement of $MgPP_i$. Now P_α of ATP is a prochiral center, so that a stereochemical investigation requires this center to be made chiral, ideally by isotopic substitution. If this could be achieved, it would be possible to determine whether amino acid activation proceeded by a direct "in line" substitution at P_α leading to inversion of configuration or whether an adenylyl–enzyme intermediate was involved on the reaction pathway. If a double-displacement mechanism was involved as advocated by Spector (1982), the expectation would be that retention of configuration at P_α would be observed.

Although the stereochemical course of the activation step of methionyl- and tyrosyl-tRNA synthetases from *E. coli* was shown to proceed with inversion of configuration at P_α with adenosine 5′-[(*S*)-α-thiotriphosphate] (Langdon & Lowe, 1979), neither the S_P nor the R_P diastereoisomers of adenosine 5′-(α-thiotriphosphate) are substrates for isoleucyl-tRNA synthetases (Von der Haar et al., 1977). It is therefore necessary, as well as desirable, to study the stereochemical course of this enzyme with ATP made chiral at P_α by isotopic substitution. The synthesis of adenosine 5′-[(*R*)-α-^{17}O]triphosphate is reported and used to investigate the stereochemical course of the activation of [$^{18}O_2$]isoleucine by isoleucyl-tRNA synthetase from *E. coli*. In addition, a positional isotope exchange study with adenosine 5′-[β-$^{18}O_2$]triphosphate is reported that further delineates the mechanism of activation of isoleucine by isoleucyl-tRNA synthetase.

Materials and Methods

Isoleucyl-tRNA synthetase from *E. coli* was obtained from Dr. C. J. Bruton (Imperial College, London) and stored in 50% glycerol containing 10 mM mercaptoethanol and 0.1 mM phenylmethanesulfonyl fluoride at −20 °C. 5′-Adenylic acid deaminase, inorganic pyrophosphatase, pyruvate kinase, adenylate kinase, hexokinase, L-amino acid oxidase, and venom phosphodiesterase (*Crotalus adamanteus*) were obtained from Sigma Chemical Co. Ltd (Poole, Dorset, U.K.). Myosin subfraction 1 was a generous gift of Dr. C. R. Bagshaw (Leicester University).

Deuterium oxide (99.8 atom % ^{2}H) was obtained from Fluorochem Ltd. (Glossop, Derbyshire, U.K.) High-grade deionized water used in the preparation of all buffers was obtained from a Milli-Q2 water purification system (Millipore Ltd., Harrow, Middlesex, U.K.). [^{18}O]Water (99 atom % ^{18}O) was obtained from Prochem Ltd. (London, U.K.).

Adenosine 5′-[(S)-α-Thiotriphosphate]. Adenosine 5′-phosphorothioate was prepared by the method of Murray & Atkinson (1968) as modified by Richards et al. (1978) and converted into adenosine 5′-[(*S*)-α-thiotriphosphate] by the combined action of adenylate kinase and pyruvate kinase (Sheu & Frey, 1977; Jaffe & Cohn, 1978).

Adenosine 5′-[(S)-α-Thiodiphosphate]. Adenosine 5′-[(*S*)-α-thiotriphosphate] was hydrolyzed to adenosine 5′-[(*S*)-α-thiodiphosphate] by both hexokinase (Stahl et al., 1974) and myosin subfraction 1 (Burgers & Eckstein, 1979).

Adenosine [β-$^{18}O_2$]Triphosphate. This was prepared by the method of Lowe & Sproat (1981) and had 95 atom % ^{18}O at the nonbridging P_β sites.

Treatment of Adenosine 5′-[(S)-α-Thiotriphosphate] with Bromine in [^{18}O]Water. Bromine (5 μL, 100 μmol) was added to a rapidly stirred solution of adenosine 5′-[(*S*)-α-thiotriphosphate] triethylammonium salt (20 μmol) in [^{18}O]water (100 μL, 50 atom % ^{18}O). After 4 min, the excess bromine was quenched with solid sodium bisulfite and the pH of the solution adjusted to 7.5 with triethylamine. The reaction mixture was diluted to 10 mL with water and applied to a column (1 cm × 10 cm) of DEAE-Sephadex[1] A-25 that had been equilibrated with triethylammonium bicarbonate buffer (50 mM, pH 7.6). The column was eluted with a linear gradient of triethylammonium bicarbonate buffer (50–600 mM, pH 7.6, 1 L). One nucleotide peak was eluted at a position that identified it as ATP (19 μmol): δ_P (H_2O, pH 9) −8.9 (d, P_γ, J = 20 Hz, $^{16}O_2$ relative intensity 0.6, $^{16}O^{18}O$ relative intensity 0.4, ^{18}O shift 2.7 Hz upfield), −14.0 (d, P_α, J = 20 Hz, $^{16}O_2$ relative intensity 1.2, $^{16}O^{18}O$ relative intensity 0.1, ^{18}O shift 3.2 Hz upfield), −24.8 (t, P_β, J = 20 Hz, relative intensity 1.0).

Preparation of Adenosine 5′-[(R)-α-^{17}O]Diphosphate. Bromine (80 μL, 1.5 mmol) was added to a rapidly stirred solution of adenosine 5′-[(*S*)-α-thiodiphosphate] triethylammonium salt (300 μmol) in [^{17}O]water (0.45 mL, 4 atom % ^{16}O, 43 atom % ^{17}O, 53 atom % ^{18}O). After 4 min, the excess bromine was quenched with solid sodium bisulfite and the pH of the solution adjusted to 7.5 with triethylamine. The reaction mixture was diluted with water (20 mL) and applied to a column (1 cm × 20 cm) of DEAE-Sephadex A-25 that had been equilibrated with triethylammonium bicarbonate buffer (50 mM, pH 7.6). The column was eluted with a linear gradient of triethylammonium bicarbonate buffer (50–400 mM, pH 7.6, 1.5 L). The appropriate fractions were pooled and evaporated to give adenosine 5′-[(*R*)-α-^{17}O]diphosphate (152 μmol): δ_P (H_2O, pH 9) −9.2 (d, P_β, J = 22.8 Hz, relative intensity 1.00), −13.7 (d, P_α, J = 22.6 Hz, $^{16}O_2$ relative intensity 0.11, $^{16}O^{18}O$ relative intensity 0.97, ^{18}O shift 3.25 Hz upfield).

Preparation of Adenosine 5′-[(R)-α-^{17}O]Triphosphate. Adenosine 5′-[(*R*)-α-^{17}O]diphosphate (72 μmol) and pyruvate kinase (230 units) were added to Tris-HCl buffer (42 mL, 42 mM, pH 7.6) containing potassium chloride (60 mM), magnesium sulfate (15 mM), phosphoenolpyruvate (3.6 mM), and bovine serum albumin (12.4 mg), and the solution was incubated at 37 °C; the pyruvate formed was assayed with lactate dehydrogenase and NADH. After 3 h, the reaction was complete. EDTA (206 mg) was added and the mixture diluted and applied to a column (1.5 cm × 30 cm) of DEAE-Sephadex A-25 equilibrated with triethylammonium bicarbonate buffer (100 mM, pH 7.6) and eluted with a linear gradient of triethylammonium bicarbonate (100–600 mM, pH 7.6, 2 L) to give adenosine 5′-[(*R*)-α-^{17}O]triphosphate (64 μmol): δ_P (H_2O, pH 9) −7.9 (d, P_γ, J = 20.0 Hz, relative intensity 1.0), −13.0 (d, P_α, J = 19.7 Hz, $^{16}O_2$ relative intensity 0.8, $^{16}O^{18}O$ relative intensity 0.1, ^{18}O shift 3.55 Hz upfield), −23.8 (t, P_β, J = 19.9 Hz, relative intensity 1.0).

Treatment of Adenosine 5′-[(S)-α-Thiodiphosphate] with Bromine in [^{18}O]Water Buffered above pH 7.6. Bromine (5 μL, 100 μmol) was added to a rapidly stirred and cooled solution of adenosine 5′-[(*S*)-α-thiodiphosphate] triethylammonium salt (20 μmol) dissolved in [^{18}O]water (35 μL) containing anhydrous sodium carbonate (6 M). After 3 min, the excess bromine was quenched with cysteine. The final pH

[1] Abbreviations: DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance.

was 7.6. The reaction mixture was diluted with water and applied to a column (1 cm × 12 cm) of DEAE-Sephadex A-25 equilibrated with triethylammonium bicarbonate buffer (50 mM, pH 7.6) and eluted with a linear gradient of triethylammonium bicarbonate buffer (50–400 mM, pH 7.6, 1 L). The [^{18}O]ADP isolated (2.8 μmol) showed the following: δ_P (H_2O, pH 9) −9.1 (d, P_β, J = 22.4 Hz, $^{16}O_2$ relative intensity 1.0, $^{16}O^{18}O$ relative intensity 0.4, ^{18}O shift 2.6 Hz upfield), −13.5 (d, P_α, J = 22.4 Hz, $^{16}O_2$ relative intensity 0.9, ^{16}O,^{18}O relative intensity 0.8, ^{18}O shift 3.3 Hz upfield).

Treatment of Adenosine 5′-[(S)-α-Thiodiphosphate] with Cyanogen Bromide in [^{18}O]Water without Buffer. Cyanogen bromide (10.6 mg, 100 μmol) in [^{18}O]water (25 μL) was added to a stirred solution of adenosine 5′-[(S)-α-thiodiphosphate] triethylammonium salt (20 μmol) in [^{18}O]water (10 μL) at 20 °C. After 5 min, cysteine was added to quench excess cyanogen bromide, followed by triethylammonium bicarbonate buffer (1 mL, 100 mM). The solution was adjusted to pH 7.6, diluted, applied to a column (1 cm × 12 cm) of DEAE-Sephadex A-25 that had been equilibrated with triethylammonium bicarbonate buffer (50 mM, pH 7.6), and eluted with a linear gradient of triethylammonium bicarbonate buffer (50–400 mM, pH 7.6, 1 L). The [^{18}O]ADP (2.2 μmol) isolated showed the following: δ_P (H_2O, pH 9) −9.1 (d, P_β, J = 22.4 Hz, $^{16}O_2$ relative intensity 1.0, $^{16}O^{18}O$ relative intensity 1.4, ^{18}O shift 2.6 Hz upfield), −13.5 (d, P_α, J = 22.4 Hz, $^{16}O_2$ relative intensity 2.0, $^{16}O^{18}O$ relative intensity 1.2, ^{18}O shift 3.3 Hz upfield).

[$^{18}O_2$]Isoleucine. Dry hydrogen chloride was bubbled through a solution of isoleucine (50 mg) in [^{18}O]water (0.5 mL, 99 atom %) for 1 min and then the solution kept at 110 °C for 72 h in a sealed tube under a nitrogen atmosphere. The [^{18}O]water was recovered by lyophilization in a vacuum line and the process repeated with [^{18}O]water (0.5 mL, 99 atom %). The [$^{18}O_2$]isoleucine (37.7 mg) was shown by mass spectrometry after methylation with diazomethane to contain 92 atom % ^{18}O and 8 atom % ^{16}O. It was possible to deduce directly from the ^{1}H NMR spectrum (since the L- and D-α-amino acids are diastereoisomers) that the [$^{18}O_2$]isoleucine was 85% L and 15% D.

Incubation of [$^{18}O_2$]Isoleucine, Adenosine 5′-[(R)-α-^{17}O]-Triphosphate, and Hydroxylamine with Isoleucyl-tRNA Synthetase. Isoleucyl-tRNA synthetase (110 μL, 4 mg/mL, 79 units/mg) and inorganic pyrophosphatase (0.43 mg, 500 units/mg) were added to Tris-HCl buffer (52 mL, 33.3 mM, pH 8) containing [$^{18}O_2$]isoleucine (1.82 mM, 92 atom % ^{18}O), adenosine 5′-[(R)-α-^{17}O]triphosphate (1.25 mM, 65 μmol), hydroxylamine hydrochloride (1 M), magnesium acetate (1.25 mM), phenylmethanesulfonyl fluoride (4.3 mg), and mercaptoethanol (20 μL), and the solution was incubated at 37 °C. The reaction was followed by assaying for residual ATP by the coupled reactions of hexokinase and glucose-6-phosphate dehydrogenase (Fromm & Zewe, 1962). When the reaction was complete (16 h), EDTA (90 μmol) was added and the solution applied to a column (1.5 cm × 30 cm) of DEAE-Sephadex A-25 that had been equilibrated with triethylammonium bicarbonate buffer (50 mM, pH 7.6) and eluted with a linear gradient of triethylammonium bicarbonate buffer (50–400 mM, pH 7.6). The [^{16}O,^{17}O,^{18}O]AMP (58 μmol) was freed from buffer by addition and evaporated of methanol (3 × 10 mL) and then cyclized and methylated for analysis by ^{31}P NMR spectroscopy of the chirality at phosphorus (Jarvest et al., 1981).

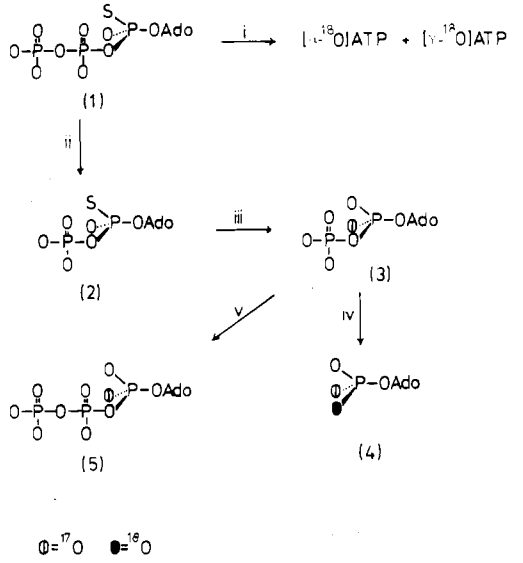
Positional Isotope Exchange with Adenosine 5′-[β-$^{18}O_2$]-Triphosphate and Isoleucyl-tRNA Synthetase. In all the experiments, Tris-HCl (12 mL, 33.3 mM, pH 8), magnesium acetate (1.25 mM), phenylmethanesulfonyl fluoride (1 mg), and 2-mercaptoethanol (5 μL) in deaerated deionized water were used. Four experiments were performed by adding the following components: (i) Isoleucine (1.8 mM), [β-$^{18}O_2$]ATP (1.25 mM), and isoleucyl-tRNA synthetase (25 μL, 4 mg/mL, 79 units/mg) were added, and the solution was incubated at 37 °C for 16.5 h. (ii) Isoleucinol hydrochloride (2.3 mM), [β-$^{18}O_2$]ATP (1.25 mM), and isoleucyl-tRNA synthetase (25 μL, 4 mg/mL, 79 units/mg) were added, and the solution was incubated at 37 °C for 16.5 h. (iii) Isoleucyl-tRNA synthetase (25 μL, 4 mg/mL, 79 units/mg) and L-amino acid oxidase (10 μL, 9.6 mg/mL, 4.7 units/mg) were added and incubated for 30 min at 37 °C. [β-$^{18}O_2$]ATP (1.25 mM) was then added and the solution incubated for a further 16.5 h at 37 °C. In a control experiment, it was shown that incubating isoleucyl-tRNA synthetase with L-amino acid oxidase did not significantly effect its activity. (iv) Two solutions were made up containing hydroxylamine hydrochloride (1M), [β-$^{18}O_2$]-ATP (1.25 mM), and isoleucyl-tRNA synthetase (25 μL, 4 mg/mL, 79 units/mg) and incubated at 37 °C for (a) 4.5 h and (b) 16.5 h. In a control experiment, it was found that the enzymic activity was reduced to 20% of the initial activity after incubation under these conditions for 16.5 h.

All the above reactions were terminated by the addition of EDTA (20 μmol, 1.7 mM) and upon vortexing with chloroform. The organic layer was washed with triethylammonium bicarbonate buffer (100 mM, pH 7.6, 3 × 2 mL) and the combined aqueous layer applied to a column (1 cm × 12 cm) of DEAE-Sephadex A-25 that had been equilibrated with triethylammonium bicarbonate buffer (100 mM, pH 7.6) and eluted with a linear gradient of triethylammonium bicarbonate buffer (100–600 mM, pH 7.6). The fractions containing [$^{18}O_2$]ATP were evaporated; addition and evaporation of methanol (3 × 5 mL) removed residual buffer. The ^{31}P NMR spectrum was measured.

Assay of Aminoacyl-tRNA Synthetases. A new assay for aminoacyl-tRNA synthetases was developed. The reaction mixture contained ATP disodium salt (1.25 mM, 15 μmol), amino acid (1.8 mM, 22 μmol), magnesium acetate (1.25 mM, 15 μmol), Tris (33.3 mM, 0.4 mmol), phenylmethanesulfonyl fluoride (1 mg), hydroxylammonium chloride (1 M, 12 mmol), and 2-mercaptoethanol (5 μL) in deionized and deaerated water (12 mL) at 37 °C. The solution was adjusted to pH 8.0, and inorganic pyrophosphatase (0.1 mg) was added followed by aminoacyl-tRNA synthetase. Aliquots (50 μL) were removed at 20-min intervals and added to trichloroacetic acid (10 μL) to denature the enzymes. Tris (1 mL, 33.3 mM, pH 6.5) was added and the solution transferred to a 1-mL quartz curvette (2-mm path length). 5′-Adenylic acid deaminase (20 μL, 0.13 mg/mL) was added to the curvette and thoroughly mixed, and the change in absorbance at 265 nm was recorded after 15 min, ΔA_{265}.

A standard curve of ΔA_{265} against percentage AMP was obtained by using four assay mixtures with varied concentrations of the sodium salts of AMP and ATP, namely: 1.25 mM AMP (7.49 mg, 15 μmol); 0.94 mM AMP (5.62 mg, 11.25 μmol) and 0.31 mM ATP (2.27 mg, 3.75 μmol); 0.625 mM AMP (3.74 mg, 7.5 μmol) and 0.625 mM ATP (4.54 mg, 7.5 μmol); 0.31 mM AMP (1.87 mg, 3.75 μmol) and 0.94 mM ATP (6.81 mg, 11.25 μmol). Aliquots (50 μL) were removed from each and treated as above to find A_{265}. The activity of the 5′-adenylic acid deaminase was also assayed after each experiment (Smiley et al., 1967) and the standard curve corrected for any change in activity.

Scheme I[a]



[a] Reagents: i, Br_2, [^{18}O]water; ii, hexokinase or myosin, Mg^{2+}; iii, Br_2, [^{17}O]water; iv, snake venom phosphodiesterase; v, phosphoenolpyruvate, pyruvate kinase, Mg^{2+}, K^+.

Comparison of ΔA_{265} with the standard curve gave the percentage AMP that was plotted against reaction time whereupon the activity was found from the initial gradient. In this assay, 1 mg of the isoleucyl-tRNA synthetase from *E. coli* produced 79 μmol of AMP/h at pH 8.0 and 37 °C.

^{31}P NMR Spectra. ^{31}P NMR spectra were recorded at 121.493 MHz on a Bruker WH-300 WB Fourier-transform spectrometer, with quadrature detection. Signal averaging was performed by an Aspect 2000 computer interfaced with the spectrometer. Field-frequency locking was provided by the deuterium resonance of D_2O or [$^2H_6$]Me_2SO. Chemical shifts (δ_P) are reported as positive when the resonances are downfield of the reference signal at 0 ppm. Chemical shifts are referred to external trimethyl phosphate in D_2O. Parameters used in Figures 1 and 2 offset 2240 Hz: sweep width 2000 Hz, acquisition time 2.05 s, pulse width 16 μs, broad-band proton coupling, Gaussian multiplication (line broadening −1.0 Hz, Gaussian broadening 0.4) in 8K and Fourier transform in 32K.

Results and Discussion

Synthesis of Adenosine 5′-[(R)-α-^{17}O]Triphosphate. The synthesis of adenosine 5′-[(*R*)-α-^{17}O]triphosphate from adenosine 5′-[(*S*)-α-thiotriphosphate] was the subject of a preliminary report (Lowe et al., 1982). As outlined in Scheme I, an attempt to convert adenosine 5′-[(*S*)-α-thiotriphosphate] into adenosine 5′-[(*R*)-α-^{18}O]triphosphate by the action of bromine in [^{18}O]water led to a mixture of [α-^{18}O]ATP and [γ-^{18}O]ATP, the latter predominating. However, treatment of adenosine 5′-[(*S*)-α-thiodiphosphate] with bromine in [^{17}O]water gave exclusively adenosine 5′-[α-^{17}O]diphosphate, which was converted to adenosine 5′-[α-^{17}O]triphosphate with phosphoenolpyruvate and pyruvate kinase. The combined effects of the nuclear electric quadrupole moment of ^{17}O on the ^{31}P signal (Lowe et al., 1979; Tsai, 1979; Tsai et al., 1980) and the ^{18}O isotope shift (Cohn & Hu, 1978; Lowe & Sproat, 1978) allowed the isotopic content at P_α to be established as 5 atom % ^{16}O, 40 atom % ^{17}O, and 55 atom % ^{18}O, in good agreement with the isotopic composition of the [^{17}O]water used in the synthesis. A portion of the adenosine 5′-[α-^{17}O]diphosphate was hydrolyzed in [^{18}O]water by *C. adamanteus* venom phosphodiesterase to adenosine 5′-[^{16}O,^{17}O,^{18}O]phosphate, which was shown to have the *S* configuration at phosphorus by our established analytical procedure (Jarvest et al., 1981). Since hydrolyses catalyzed by snake venom phosphodiesterase proceed with retention of configuration at phosphorus (Jarvest & Lowe, 1981; Mehdi & Gerlt, 1981), the adenosine 5′-[α-^{17}O]diphosphate must be the R_P isomer. Comparison of the observed relative intensities of the ^{31}P resonances in Figure 1 with those calculated for substitution with inversion of configuration, however, revealed that a small amount of racemization has occurred; the best fit of the data indicates that the reaction proceeded with 93% inversion and 7% retention of configuration. In a subsequent preparation, the reaction proceeded stereospecifically within experimental error.

The substitution of sulfur by ^{18}O in nucleoside phosphorothioates has also been achieved with *N*-bromosuccinimide in dioxane–[^{18}O]water (Connolly et al., 1982). However, when adenosine 5′-[(*S*)-α-thiodiphosphate] is treated with cyanogen bromide in [^{18}O]water at pH 10.6, the [^{18}O]ADP that is isolated has ^{18}O in both P_α and P_β (Sammons et al., 1982), and because of this, a more elaborate synthesis by way of adenosine 5′-[1-thio-2-(cyanoethyl)diphosphate] was developed (Sammons & Frey, 1982). Sammons et al. (1982) postulated the intervention of a cyclo diphosphate intermediate in the reaction of adenosine 5′-[(*S*)-α-thiodiphosphate] with cyanogen bromide. Similar observations were made with adenosine 5′-(β-thiotriphosphate).

The different behavior of adenosine 5′-[(*S*)-α-thiodiphosphate] toward cyanogen bromide and bromine (or *N*-bromosuccinimide) in [^{18}O]water seemed likely to be due to one or both of two possible causes. It is possible that the leaving ability of the group (of uncertain structure) generated by the action of bromine (or *N*-bromosuccinimide) in water on adenosine 5′-[(*S*)-α-thiodiphosphate] is much better than that of the thiocyanate group postulated in the intermediate of the cyanogen bromide reaction, so that participation by the β-phosphate is incapable of providing assistance to the departing group. Alternatively, in the cyanogen bromide reaction, the pH of the solution had been maintained at 10.6 by potassium tetraborate whereas in the bromine–water (or *N*-bromosuccinimide–water) reaction buffer had not been used and the reaction mixture became acidic. Under these conditions, the β-phosphate would bear only one charge and as such would be expected to be less able to participate in the reaction. When adenosine 5′-[(*S*)-α-thiodiphosphate] was treated with bromine in [^{18}O]water in sodium carbonate buffer, the [^{18}O]ADP isolated was shown by ^{31}P NMR spectroscopy to be labeled in both P_β and P_α in the ratio of about 1:2. Thus, by maintaining P_β in its dianionic state, participation by P_β does compete with direct nucleophilic substitution. When adenosine 5′-[(*S*)-α-thiodiphosphate] was treated with cyanogen bromide in [^{18}O]water in the absence of buffer, the [^{18}O]ADP was labeled about equally at P_β and P_α. It would seem therefore that both the state of ionization of P_β and the leaving ability of the modified sulfur moiety are factors that determine whether neighboring-group participation by P_β competes with direct nucleophilic substitution. With the more modest leaving ability of the group generated by treatment of adenosine 5′-[(*S*)-α-thiodiphosphate] with cyanogen bromide, protonation of P_β is insufficient to suppress participation, and esterification is required. Protonation or esterification of P_β also suppresses the spontaneous decomposition of the activated intermediates.

Since P^1,P^1-disubstituted pyrophosphate dianions rapidly decompose to phosphate diesters and inorganic phosphate

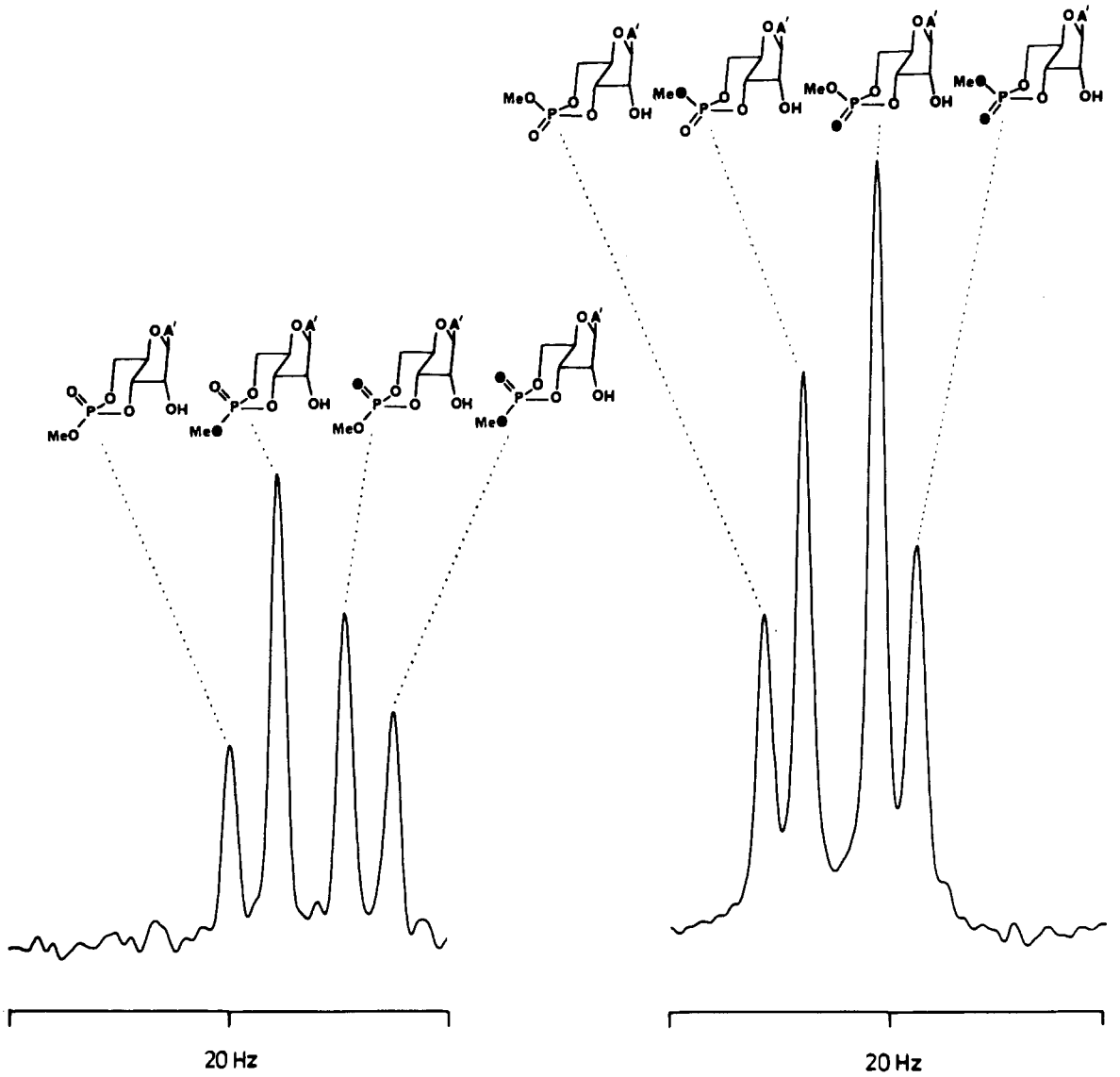
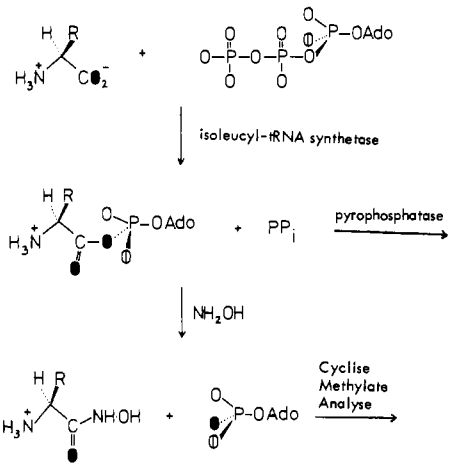


FIGURE 1: ^{31}P NMR spectrum (121.5 MHz) of equatorial and axial triesters derived by cyclization and methylation of 5′-[^{16}O,^{17}O,^{18}O]AMP obtained by snake venom phosphodiesterase catalyzed hydrolysis of adenosine 5′-[α-^{17}O]diphosphate in [^{18}O]water. The ratio of the $^{16}O_{ax}$,$^{18}O_{eq}$ to $^{18}O_{ax}$,$^{16}O_{eq}$ triesters shows that the 5′-[^{16}O,^{17}O,^{18}O]AMP has the S_P configuration and, hence, the adenosine 5′-[α-^{17}O]diphosphate has the R_P configuration as shown in Scheme I. (●) ^{18}O; (A′) 1-methyladenine.

Scheme II: Stereochemical Course of Activation of [$^{18}O_2$]Isoleucine by Adenosine 5′-[(*R*)-α-^{17}O]Triphosphate and Isoleucyl-tRNA Synthetase from *E. coli*[a]



[a] The [^{17}O,$^{18}O_2$]isoleucyl adenylate was cleaved in situ by hydroxylamine. The evidence for the R_P configuration of the 5′-[^{16}O,^{17}O,^{18}O]AMP is provided in Figure 2.

(Brown & Hamer, 1960; Samuel & Silver, 1961), above the second pK_a of the β-phosphate of the activated intermediates of adenosine 5′-[(*S*)-α-thiodiphosphate] spontaneous decomposition would be expected, leading (ultimately) to AMP and P_i, which accounts for the poor yields of [^{18}O]ADP obtained under these conditions.

Although the synthesis of a nucleoside 5′-[(*S*)-α-^{18}O]diphosphate from [(R_P)-^{18}O]-cyclic-dAMP with adenylate cyclase (*Brevibacterium liquefaciens*) and glycerol kinase has been described (Coderre & Gerlt, 1980), the ready availability of nucleoside 5′-(α-thiodiphosphates) (Eckstein, 1975, 1979) makes the direct displacement of sulfur by an oxygen isotope (as described above) a more versatile and attractive route.

Stereochemistry Course of Activation of Isoleucine by Isoleucyl-tRNA Synthetase. [$^{18}O_2$]Isoleucine and adenosine 5′-[(*R*)-α-^{17}O]triphosphate were incubated with isoleucyl-tRNA synthetase from *E. coli* in the presence of Mg^{2+} and hydroxylamine. Inorganic pyrophosphatase was also present in the reaction mixture to hydrolyze the magnesium pyrophosphate generated and so assist the overall reaction that is outlined in Scheme II. The [^{16}O,^{17}O,^{18}O]AMP was isolated

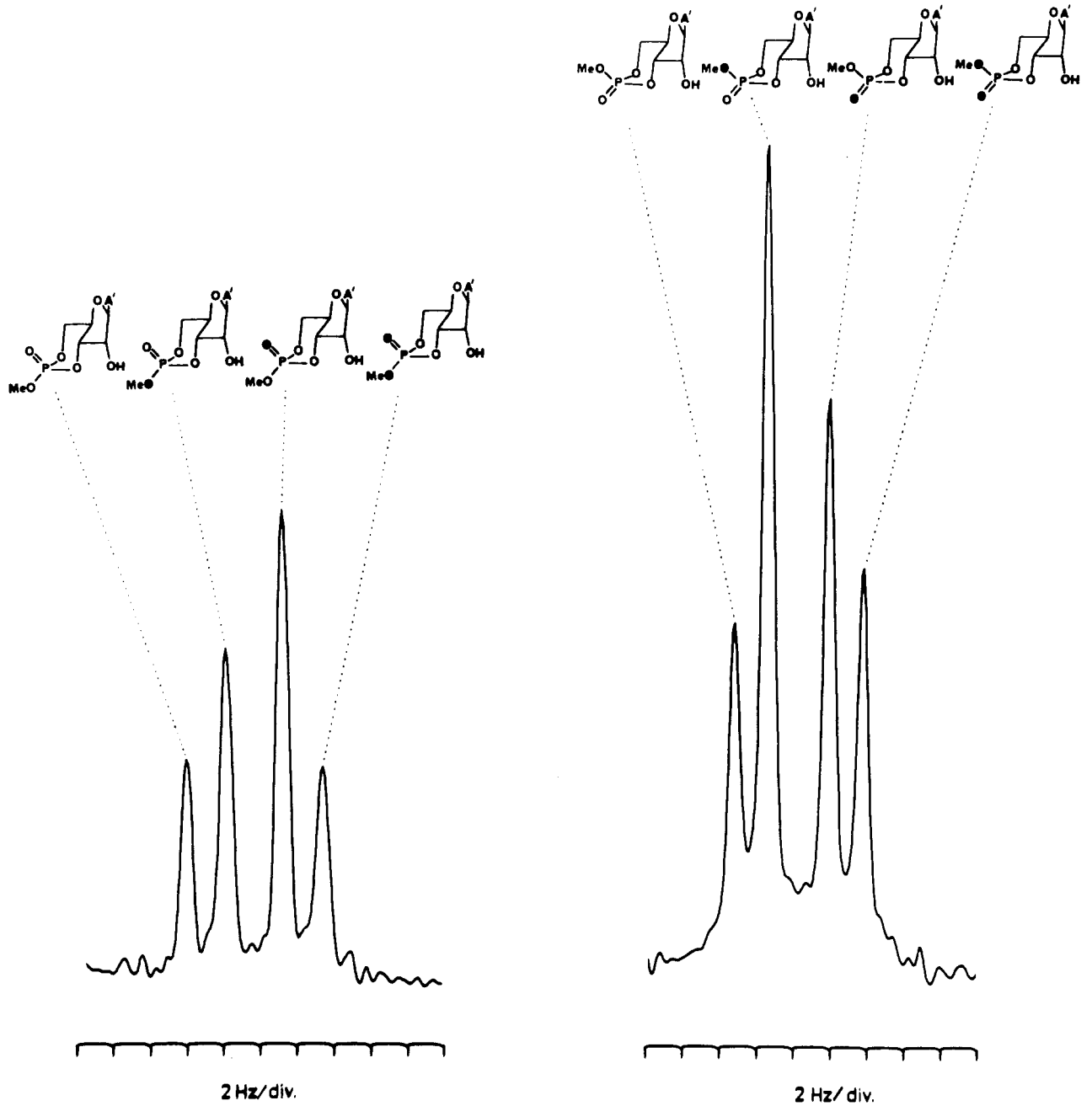


FIGURE 2: ^{31}P NMR spectrum (121.5 MHz) of equatorial and axial triesters derived by cyclization and methylation of 5′-[^{16}O,^{17}O,^{18}O]AMP obtained by incubating [$^{18}O_2$]isoleucine, adenosine 5′-[(*R*)-α-^{17}O]triphosphate, hydroxylamine, and isoleucyl-tRNA synthetase. The ratio of the $^{16}O_{ax}$,$^{18}O_{eq}$ to $^{18}O_{ax}$,$^{16}O_{eq}$ triesters shows that the 5′-[^{16}O,^{17}O,^{18}O]AMP has the R_P configuration and, hence, reaction has proceeded with inversion of configuration at P_α of ATP as indicated in Scheme II. (●) ^{18}O; (A′) 1-methyladenine.

from the reaction mixture and its chirality at phosphorus determined by our established procedure after cyclization and methylation (Jarvest et al., 1981). The ^{31}P NMR spectrum is shown in Figure 2. From the known isotopic content of the isoleucine and the isotopic content and enantiomeric excess of the adenosine 5′-[(*R*)-α-^{17}O]triphosphate used in this reaction, it was possible to calculate the expected relative peak intensities of the ^{31}P NMR resonances of the equatorial and axial triesters, for the reaction proceeding with retention and inversion of configuration. Comparison of the observed and calculated relative peak intensities shows that the activation of [$^{18}O_2$]isoleucine by adenosine 5′-[(*R*)-α-^{17}O]triphosphate occurs stereospecifically (within experimental error) with inversion of configuration at P_α. This is most simply interpreted in terms of a direct in line displacement of pyrophosphate from P_α of ATP by isoleucine and effectively excludes the possibility of a double-displacement mechanism involving an adenylyl–enzyme intermediate.

Effect of Isoleucine-tRNA Synthetase on [β-$^{18}O_2$]ATP. The observation that isoleucyl-tRNA synthetase catalyzes the formation of isoleucyl adenylate from isoleucine and MgATP with inversion of configuration at phosphorus actually implies that an odd number of displacement reactions have occurred at phosphorus, the simplest being one, since there is now ample evidence that single enzyme-catalyzed displacement reactions at phosphate esters and anhydrides occur with inversion of configuration at phosphorus (Knowles, 1980; Lowe et al., 1981). In view of the evidence for an adenylyl–enzyme intermediate when tryptophanyl-tRNA synthetase (from beef pancreas) is incubated with MgATP (Kiselev & Kochkina, 1973) and the suggestion that this implies that adenylyl–enzyme intermediates may be on the reaction pathway for all

aminoacyl-tRNA synthetases (Spector, 1982), we have undertaken experiments with isoleucyl-tRNA synthetase to explore the possibility that several adenylyl–enzyme intermediates (any even number would be consistent with inversion of configuration at phosphorus) are on the reaction pathway.

The availability of adenosine 5′-[β-$^{18}O_2$]triphosphate ([β-$^{18}O_2$]ATP) (Lowe & Sproat, 1981) allows a subtle test to be applied for the formation of an adenylyl–enzyme intermediate, since its lifetime need only be sufficient to allow rotation about the O–P$^{18}O_2$ bond in the [β-$^{18}O_2$]pyrophosphate for positional isotope exchange (Rose, 1979) to occur leading to ^{18}O appearing in the P_α–O–P_β bridge of recovered [$^{18}O_2$]ATP.

Initially, isoleucyl-tRNA synthetase was incubated with isoleucine and [β-$^{18}O_2$]ATP in the presence of Mg^{2+} for 16.5 h at 37 °C. As expected, the label was completely scrambled by torsional rotation (leading to ^{18}O in the P_α–O–P_β bridge) and by tumbling (leading to $^{18}O_2$ at P_γ). In the absence of added isoleucine, incubation of isoleucyl-tRNA synthetase with [β-$^{18}O_2$]ATP for 16.5 h under otherwise identical conditions led to partial scrambling, but this was due to traces of endogenous isoleucine present in the enzyme preparation, since if the enzyme was preincubated with L-amino acid oxidase (the isoleucyl-tRNA synthetase was still active after this treatment) prior to addition of the [β-$^{18}O_2$]ATP, no positional isotope exchange occurred after 16.5 h at 37 °C. In a further control experiment, it was found that if hydroxylamine was added to the isoleucyl-tRNA synthetase prior to the [β-$^{18}O_2$]ATP, partial scrambling occurred initially (investigated at 4.5 h), but thereafter, no further scrambling occurred up to 16.5 h (the enzyme was shown to be active at the end of this period). The initial scrambling was expected since activation of the endogenous isoleucine is necessary before it can react with hydroxylamine to form isoleucylhydroxamic acid. Finally, in the absence of added isoleucine but in the presence of the competitive inhibitor isoleucinol, no positional isotope exchange occurred in [β-$^{18}O_2$]ATP up to 16.5 h.

These results show that an adenylyl intermediate is not formed between ATP and isoleucyl-tRNA synthetase in the absence of isoleucine or in the presence of isoleucinol (2.3 mM), which is a very effective competitive inhibitor (K_i of 3.3 μM) of isoleucine in the presence of MgATP with which it exhibits synergistic binding (Holler et al., 1975). Thus multiple odd numbered displacements at P_α of ATP can be excluded, leaving the direct in line displacement of pyrophosphate from ATP by isoleucine as the only acceptable interpretation of the stereochemical course of the reaction. The lack of positional isotope exchange in the absence of isoleucine and in the presence of isoleucinol also provides evidence against the dissociative mechanism although this was not considered likely since there is no chemical precedent for such a mechanism with a phosphate diester. We therefore conclude that isoleucyl-tRNA synthetase catalyzes the activation of isoleucine by a direct associative in line displacement at P_α of ATP.

Acknowledgments

We are grateful to Professor Perry A. Frey for information prior to publication on the reaction between adenosine 5′-[(*S*)-α-thiodiphosphate] and cyanogen bromide at pH 10.6.

Registry No. **1**, 58976-48-0; **2**, 59286-20-3; **3**, 83541-22-4; **5**, 83541-23-5; adenosine 5′-[β-$^{18}O_2$]triphosphate, 79154-58-8; isoleucyl-tRNA synthetase, 9030-96-0.

References

Arndt, D. J., & Berg, P. (1970) *J. Biol. Chem. 245*, 665.
Baldwin, A. N., & Berg, P. (1966) *J. Biol. Chem. 241*, 831.
Berthelot, F., & Yaniv, M. (1970) *Eur. J. Biochem. 16*, 123.
Brown, D. M., & Hamer, N. K. (1960) *J. Chem. Soc.*, 1155.
Burgers, P. M. J., & Eckstein, F. (1979) *Biochemistry 18*, 450.
Coderre, J. A., & Gerlt, J. A. (1980) *J. Am. Chem. Soc. 102*, 6594.
Cohn, M., & Hu, A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 200.
Connolly, B. A., Eckstein, F., & Fülder, H. H. (1982) *J. Biol. Chem. 257*, 3382.
Durekovic, A., Flossdorf, J., & Kula, M.-L. (1973) *Eur. J. Biochem. 36*, 528.
Eckstein, F. (1975) *Angew. Chem., Int. Ed. Engl. 14*, 160.
Eckstein, F. (1979) *Acc. Chem. Res. 12*, 204.
Fersht, A. R. (1981) *Proc. R. Soc. London, Ser. B 212*, 351.
Fromm, H. J., & Zewe, V. (1962) *J. Biol. Chem. 237*, 3027.
Holler, E., Hammer-Raber, B., Hanke, T., & Bartmann, P. (1975) *Biochemistry 14*, 2496.
Jaffe, E. K., & Cohn, M. (1978) *Biochemistry 17*, 652.
Jarvest, R. L., & Lowe, G. (1981) *Biochem. J. 199*, 447.
Jarvest, R. L., Lowe, G., & Potter, B. V. L. (1981) *J. Chem. Soc., Perkin Trans. 1*, 3186.
Kalousek, F., & Konigsberg, W. (1976) *FEBS Lett. 61*, 151.
Kim, J.-J. P., Chakraburtty, K., & Mehler, A. H. (1977) *J. Biol. Chem. 252*, 2698.
Kiselev, L. L., & Kochkina, L. L. (1973) *Doklady Akad. Nauk SSSR 214*, 215; (1974) *Dokl. Biochem. (Engl. Transl.) 214*, 7.
Kiselev, L. L., & Favorova, O. O. (1974) *Adv. Enzymol. Relat. Areas Mol. Biol. 40*, 141.
Knowles, J. R. (1980) *Annu. Rev. Biochem. 49*, 877.
Koch, G. L. E., Boulanger, Y., & Hartley, B. S. (1974) *Nature (London) 249*, 316.
Kula, M.-R. (1973) *FEBS Lett. 35*, 299.
Langdon, S. P., & Lowe, G. (1979) *Nature (London) 281*, 320.
Lowe, G., & Sproat, B. S. (1978) *J. Chem. Soc., Chem. Commun.*, 565.
Lowe, G., & Sproat, B. S. (1981) *J. Chem. Soc., Perkin Trans. 1*, 1874.
Lowe, G., Potter, B. V. L., Sproat, B. S., & Hull, W. E. (1979) *J. Chem. Soc., Chem. Commun.*, 733.
Lowe, G., Cullis, P. M., Jarvest, R. L., Potter, B. V. L., & Sproat, B. S. (1981) *Philos. Trans. R. Soc. London, Ser. B 293*, 75.
Lowe, G., Tansley, G., & Cullis, P. M. (1982) *J. Chem. Soc., Chem. Commun.*, 595.
Mehdi, S., & Gerlt, J. A. (1981) *J. Biol. Chem. 256*, 12164.
Murray, A. W., & Atkinson, R. (1968) *Biochemistry 7*, 4023.
Richards, J. P., Ho, H.-T., & Frey, P. A. (1978) *J. Am. Chem. Soc. 100*, 7756.
Rose, I. A. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 50*, 361.
Sammons, R. D., & Frey, P. A. (1982) *J. Biol. Chem. 257*, 1138.
Sammons, R. D., Ho, H.-T., & Frey, P. A. (1982) *J. Am. Chem. Soc. 104*, 5841.
Samuel, D., & Silver, B. (1961) *J. Chem. Soc., 4321.*
Schimmel, P. R., & Söll, D. (1979) *Annu. Rev. Biochem. 48*, 601.
Sheu, K.-F. R., & Frey, P. A. (1977) *J. Biol. Chem. 252*, 4445.
Smiley, K. L., Jr., Berry, A. J., & Suelter, C. H. (1967) *J. Biol. Chem. 242*, 2502.

Söll, D., & Schimmel, P. R. (1974) *Enzymes, 3rd Ed. 10*, 489.
Spector, L. B. (1982) *Covalent Catalysis by Enzymes*, p 202, Springer-Verlag, New York.
Stahl, K. W., Schlimme, E., & Eckstein, F. (1974) *FEBS Lett. 40*, 241.
Tsai, M.-D. (1979) *Biochemistry 18*, 1468.
Tsai, M.-D., Huang, S. L., Kozlowski, J. F., & Chang, C. C. (1980) *Biochemistry 19*, 3531.
Von der Haar, F., Cramer, F., Eckstein, F., & Stahl, K.-W. (1977) *Eur. J. Biochem. 76*, 263.
Waterson, R. M., & Konigsberg, W. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 376.

Stoichiometry of Sodium- and Chloride-Coupled γ-Aminobutyric Acid Transport by Synaptic Plasma Membrane Vesicles Isolated from Rat Brain†

Rodica Radian and Baruch I. Kanner*

ABSTRACT: Transport of γ-aminobutyric acid (GABA) into synaptic plasma membrane vesicles exhibits an absolute dependency on both sodium and chloride. The requirement for chloride is not due to its ability to serve as a permeant anion. Chloride ion does not merely fulfill a need for a permeant ion since GABA accumulation still requires external chloride when a K^+ diffusion potential (interior negative) is imposed across the vesicle membrane with valinomycin. K_m is lowered and V_{max} is raised by either sodium or chloride ions. A plot of the logarithm of the concentration ratio of GABA (internal/external) at steady state vs. the logarithm of the concentration ratio of sodium or chloride ions (both external/internal) yields straight lines with slopes of 1.50 ± 0.20 or 0.47 ± 0.02, respectively. Both GABA and tetraphenylphosphonium ion transport are affected to a similar extent by either valinomycin (enhanced) or carbonyl cyanide *m*-chlorophenylhydrazone (inhibited). In the presence of K^+/valinomycin a plot of the logarithm of the concentration ratio of GABA (internal/external) at steady state vs. the logarithm of the concentration ratio of potassium (internal/external) yields a straight line with a slope of 0.90 ± 0.08. The simplest stoichiometry for the translocation cycle catalyzed by the GABA transporter is the influx of two sodium ions and one chloride ion per GABA zwitterion.

Membrane vesicles isolated from various bacterial and mammalian cells have proved extremely useful for the study of active transport [cf. Kaback (1974), Aronson & Sactor (1974), Hopfer et al. (1973), Colombini & Johnstone (1974), Lever (1977), and Rudnick (1977)]. Some of their advantages include the possibility of using well-defined energy sources and the lack of metabolism and storage in subcellular organelles. Recently, the use of membrane vesicles has been extended to the study of the synaptic plasma membrane (Kanner, 1980) for the investigation of sodium-dependent neurotransmitter transport in rat brain (Kanner, 1978; Kanner & Sharon, 1978). These transport systems have been implicated in the termination of transmitter action on postsynaptic receptors (Iversen, 1971).

Using the synaptic plasma membrane vesicles, it has been shown that the general concept that solute accumulation can be achieved by cotransport with ions (Crane, 1965; Riggs et al., 1958; Mitchell, 1963) also applies to neurotransmitters in the brain. Thus, the electrochemical potential gradient of Na^+ serves as a direct driving force for the transport of GABA[1] (Kanner, 1978) and L-glutamic acid (Kanner & Sharon, 1978). Surprisingly, these studies revealed that neurotransmitter transport is absolutely dependent on additional ions, such as external Cl^- or small monovalent anions in the case of GABA (Kanner, 1978) and internal K^+ in the case of L-glutamate (Kanner & Sharon, 1978). Recent experiments have provided strong evidence that the GABA transporter catalyzes influx of GABA coupled with the influx of both sodium and chloride ions (Kanner & Kifer, 1981). Similarly, it appears that the L-glutamate transporter catalyzes influx of L-glutamate coupled with the influx of sodium ions and the efflux of potassium ions (Kanner & Marva, 1982). Other recent examples of participation of ions in addition to sodium in the translocation cycle of solute transport systems include the serotonin transporter from platelets (Nelson & Rudnick, 1979; Nelson & Rudnick, 1982) and the L-glutamate transporter from renal brush border vesicles (Burckhardt et al., 1980; Schneider & Sacktor, 1980; Sacktor et al., 1981).

The studies described in this paper focus on the stoichiometry of the GABA transporter. Because of the electrogenicity of the transporter (Kanner, 1978), it is predicted that the stoichiometry will be nNa$^+$:mCl$^-$:GABA with $n > m$. Studies on the sodium dependence of GABA transport in intact synaptosomes indicate a positive cooperativity for sodium ions with a Hill coefficient of slightly over (Martin & Smith, 1972) or under 2 (Blaustein & King, 1976). Recently, similar studies have indicated that the Hill coefficient for the chloride dependence is about 1 (Kuhar & Zarbin, 1978). These observations are also consistent with $n > m$.

This paper describes a thermodynamic approach to study the stoichiometry of the process in synaptic plasma membrane vesicles. It is concluded that the stoichiometry for the translocation cycle catalyzed by the GABA transporter is the

† From the Laboratory of Neurochemistry, Institute of Biochemistry, Hadassah Medical School, The Hebrew University, Jerusalem, Israel. *Received February 22, 1982; revised manuscript received October 11, 1982.* Supported by Grant NS 16708-01 from the National Institute of Neurology and Communicative Disorders and Stroke and by Grant 2152/80 of the U.S.–Israel Binational Science Foundation.

[1] Abbreviations: GABA, γ-aminobutyric acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TPP^+, tetraphenylphosphonium ion; $\Delta\tilde{\mu}_{Na^+}$, (RT/F) ln $([Na^+]_{out}/[Na^+]_{in}) - \Delta\psi$; $\Delta\psi$, membrane potential (outside is zero); $\Delta\tilde{\mu}_{Cl^-}$, (RT/F) ln $([Cl^-]_{out}/[Cl^-]_{in}) + \Delta\psi$.

0006-2960/83/0422-1236$01.50/0