salt solutions of 100–200 mM, the counterion concentration is ample to neutralize the charge on most proteins. At significantly lower counterion concentrations, there is the potential for large electrostatic effects that could play a critical role in stabilizing the three-dimensional structure. In addition, in low ionic strength environments the motions of counterions will be coupled with those of the macromolecule, and this could affect hydrodynamic measurements, such as in the compressibility experiments.

Whether the increase in radius of gyration observed for oxidized cytochrome *c* in the low ionic strength solution environment is due to partial unfolding of the structure or some alternative rearrangement, it is clear that under the solution conditions used for the compressibility measurements there is a real structural difference between oxidized and reduced cytochrome *c*. This difference is not evident in the crystal structures because the crystals were obtained from high ionic strength solutions. The structural change observed at low ionic strength in solution is likely to be accompanied by a change in the low-frequency dynamics, and it is likely that it is a combination of changes in time-averaged structure as well as in the structural dynamics that gives rise to the different hydrogen exchange rates and apparent compressibilities between the two forms.

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A New Synthesis of Adenosine 5′-([$\gamma(R)$-^{17}O,^{18}O]-γ-Thiotriphosphate) and Its Use To Determine the Stereochemical Course of the Activation of Glutamate by Glutamine Synthetase†

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ABSTRACT: A new synthetic route to adenosine 5′-([$\gamma(R)$-^{17}O,^{18}O]-γ-thiotriphosphate) is described which combines chemical methods for introducing the heavy oxygen isotopes and enzymic methods for achieving the enantiospecificity. This material was used as a substrate for the activation of glutamate catalyzed by glutamine synthetase from *Salmonella typhimurium*. Analysis of the chirality of the [^{16}O,^{17}O,^{18}O]thiophosphate produced showed that the reaction proceeds with inversion of configuration on phosphorus. This result, taken together with the positional isotope exchange studies of Midelfort and Rose [Midelfort, C. F., & Rose, I. A. (1976) *J. Biol. Chem. 251*, 5881–5887], demonstrates that the activation of glutamate to form γ-glutamyl phosphate proceeds by a direct "in-line" transfer of the phosphoryl group.

Glutamine synthetase [L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2] catalyzes the conversion of glutamate to glutamine and is responsible for the assimilation of ammonia. Since glutamine is the biosynthetic source of nitrogen for amino acids, purines, pyrimidines, and many other molecules essential for cellular activity, its enzymic activity is regulated in a complex way by many cellular metabolites, by adenylylation and deadenylylation, and by a closed bicyclic cascade mechanism (Rhee et al., 1985). However, there are considerable differences between the glutamine synthetases from different species. The prokaryotic enzymes are usually dodecameric (Valentine et al., 1968; Shapiro & Ginsburg, 1968), while eukaryotic enzymes are octameric (Meister, 1985). The unadenylylated glutamine synthetase from *Salmonella typhimurium*, whose crystal structure was reported recently at 3.5-Å resolution (Almassey et al., 1986), is the

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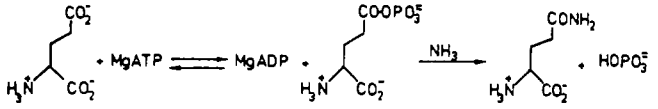
Scheme I: Minimal Pathway for Glutamine Synthetase Catalyzed Reaction



material used in this investigation.

Although the prokaryotic and eukaryotic enzymes differ markedly in their physical properties, they are thought to have a common chemical mechanism (Meister, 1985; Rhee et al., 1985). The first step is believed to be the activation of glutamate with the formation of γ-glutamyl phosphate, which may occur before or after the binding of ammonia (Clark & Villafranca, 1985). A wealth of evidence has been reported in support of γ-glutamyl phosphate as an intermediate, and its chemical and kinetic competencies have been demonstrated (Midelfort & Rose, 1976; Meek et al., 1982). The enzyme-bound γ-glutamyl phosphate subsequently reacts with bound ammonia, completing the overall reaction (Scheme I).

In order to study the stereochemical course of enzymic reactions which lead to the production of inorganic phosphate, it is necessary to use thiophosphoryl analogues of the natural substrates chirally labeled with oxygen isotopes (Webb, 1982); it is also necessary to be able to analyze chiral inorganic [^{16}O,^{17}O,^{18}O]thiophosphate. To this end, we have recently developed a new stereochemical analysis of chiral inorganic [^{16}O,^{17}O,^{18}O]thiophosphate (Arnold & Lowe, 1986; Arnold et al., 1987), which allows for a more quantitative measurement of the chirality than the original method (Webb & Trentham, 1980; Tsai & Chang, 1980).

The earliest work in which chiral [^{16}O,^{17}O,^{18}O]thiophosphate was stereochemically analyzed made use of an existing method for the synthesis of adenosine 5′-([γ(R)-^{16}O,^{18}O]-γ-thiotriphosphate) (Richard & Frey, 1978). The ATPase reactions were conducted in [^{17}O]water, thereby producing chiral inorganic [^{16}O,^{17}O,^{18}O]thiophosphate (Webb & Trentham, 1980). Subsequently, by introduction of ^{18}O into the adenosine used as the starting material for this synthesis, adenosine 5′-([γ(S)-^{17}O,^{18}O]-γ-thiotriphosphate) was made, obviating the need for large quantities of [^{17}O]water in the enzymic reaction (Webb & Trentham, 1981; Webb, 1982). This also facilitates the determination of the stereochemical course of other reactions in which the final oxygen to be incorporated into the thiophosphate does not come from water. However, difficulties in repeating the synthesis of labeled adenosine have been encountered (P. A. Frey, personal communication), but after the completion of this work, which has been reported in a preliminary form (Bethell & Lowe, 1986), a modification of the original procedure was reported. Although the modified procedure now establishes a reproducible protocol for the synthesis of the intermediate [5′-^{18}O]adenosine, the incorporation of isotope is extremely inefficient, only 1.2 mmol of [5′-^{18}O]adenosine being produced from 175 mmol of $H_2{}^{18}O$, and moreover, the isotope enrichment achieved is only 75% (from 95% enriched $H_2{}^{18}O$) (Iyengar et al., 1986). The new synthesis of adenosine 5′-([γ(R)-^{17}O,^{18}O]-γ-thiotriphosphate) reported here is a significant improvement in overall yield, efficiency of isotope incorporation, and isotope enrichment. This material has been used to study the stereochemical course of activation of glutamate by glutamine synthetase from *S. typhimurium*.

Materials and Methods

Glutamine synthetase (*Salmonella typhimurium*), in the unadenylylated state (Janson et al., 1984), was a gift from Prof. D. Eisenberg (University of California). It was assayed by the γ-glutamyl hydroxamate/arsenate method (Shapiro & Stadtman, 1970) and had an activity of 160 units/mL, 1 unit producing 1 μmol of γ-glutamyl hydroxyamate in 1 min at 37 °C. Methionyl-tRNA synthetase (from *Bacillus stearothermophilus*) (84 units mg^{-1} and 8 mg mL^{-1}) was obtained from Dr. C. Bruton (Imperial College, London). Pyruvate kinase and hexokinase were obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, U.K.). High-grade deionized water used in the preparation of all buffers was obtained from a Milli-Q2 water purification system. [^{18}O]water (99.0 atom % ^{18}O) was obtained from Amersham International plc (Amersham, U.K.), and [^{17}O]water (23.5 atom % ^{16}O, 55.9 atom % ^{17}O, 20.6 atom % ^{18}O) was obtained from Monsanto Research Corp. (Miamisburg, OH).

Analysis of nucleotides was performed by ion-exchange chromatography with an FPLC system (Pharmacia, Hounslow, U.K.) and Mono Q resin. The column was eluted with Tris-HCl[1] buffer (10–1000 mM, pH 8.0, 20 °C), the buffer composition for elution being ADPβS (510 mM), ATPβS (550 mM), and ATPγS (590 mM). pH measurements were conducted with a Radiometer PHM84 pH meter. ^{1}H NMR spectra were recorded on a Bruker WM300 at 300 MHz and on a Bruker AM500 at 500 MHz and were referenced to external tetramethylsilane. ^{31}P NMR spectra were recorded on a Bruker AM250 operating at 101.26 MHz with a multinuclear probe and on a Bruker AM500 operating at 202.46 MHz with a phosphorus-dedicated probe and were referenced to external trimethyl phosphate in D_2O. Ultraviolet absorption measurements were conducted on a Pye-Unicam SP8-100 spectrometer. Melting points were measured on a Kofler block and are uncorrected. All DEAE-Sephadex A-25 columns were used at 4 °C, whereas the Mono Q column for FPLC was run at ambient temperature. Product-containing fractions were determined by UV absorption and/or the $PdCl_2$ spray test for sulfur-containing compounds.

Bis(4-nitrobenzyl) Disulfide. This was prepared by the general method of Gladysz et al. (1979). Powdered sulfur (0.32 g, 0.01 mol) was placed in a dry flask under nitrogen, and a 1.0 M solution of lithium triethylborohydride in tetrahydrofuran (10 mL) was added, with stirring, over 10 min, the resulting mixture being left for 15 min. 4-Nitrobenzyl bromide (2.16 g) in tetrahydrofuran (10 mL) was added dropwise and the mixture stirred for a further 4 h. The reaction mixture was partitioned between ether (100 mL) and water (100 mL), the aqueous layer was extracted further with ether (2 × 50 mL), and the combined ether extracts were evaporated under reduced pressure. The disulfide was recrystallized from ethanol and water to give pale yellow-orange crystals of the disulfide: 0.76 g, 45%; mp 122–123 °C [Milligan and Swan (1962) give mp 126.5 °C]; ^{1}H NMR ($CDCl_3$) δ 8.2 (d, J = 8.7 Hz, 2 H), 7.4 (d, J = 8.7 Hz, 2 H, [C_6H_4] A_2B_2 system), 3.7 (s, 2 H, [CH_2S]); mass spectrum (DCI, NH_3), m/z (relative intensity) 354 (M + 18) (100).

S-(4-Nitrobenzyl) [$^{17}O_3$]Phosphorothioate. This was prepared by the method of Bethell and Lowe (1987). Phosphorus trichloride (175 μL, 2 mmol) was placed in a dry flask under nitrogen, and to it was added [^{17}O]water (171 μL) dropwise. The reaction was stirred for 30 min at room temperature, for

[1] Abbreviations: AMP, adenosine 5′-monophosphate; AMPS, adenosine 5′-phosphorothioate; ADPβS, adenosine 5′-(β-thiodiphosphate); ATPβS, adenosine 5′-(β-thiotriphosphate); ATPγS, adenosine 5′-(γ-thiotriphosphate); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

1 h at 50 °C, and then at room temperature overnight. The resulting phosphorous acid was dried by the addition and evaporation of dry pyridine (3 × 10 mL). The residue was dissolved in pyridine (20 mL), and to the stirred solution was added triethylamine (0.92 mL) and chlorotrimethylsilane (0.84 mL). The mixture was stirred for 10 min. Bis(4-nitrobenzyl) disulfide (0.68 g) was then added and the mixture stirred for 18 h. The mixture was filtered, and to the filtrate was added water (4 mL). The solution was evaporated and the residue dissolved in sodium hydroxide solution (0.26 g in 50 mL). The solution was diluted and applied to a column of DEAE-Sephadex A-25 (150 mL). The column was eluted with triethylammonium bicarbonate buffer (30–300 mM, pH 8.0) at 90 mL h^{-1} over 24 h, 18-mL fractions being collected. The first six product-containing fractions (eluted at 175 mM buffer) were contaminated by a small quantity of inorganic thiophosphate which was removed by subjecting it again to the same chromatographic procedure. The product-containing fractions were combined and evaporated under reduced pressure. Residual triethylammonium bicarbonate was removed by the repeated evaporation of aliquots of methanol (20 mL) to give the product: 0.48 g, 1.2 mmol, 60%; ^{31}P NMR (D_2O) δ 12.3 (^{18}O shift = 0.027 ppm per ^{18}O).

Adenosine 5′-[$^{18}O_3$]Monophosphate. This was prepared by the method of Bethell and Lowe (1987) and contained 94 atom % ^{18}O per site.

Adenosine 5′-[S-(4-Nitrobenzyl) [α,α-$^{18}O_2$,$\alpha\beta$-^{18}O,β,β-$^{17}O_2$]-β-thiodiphosphate]. The compound was made by a modification of the general method of Michelson (1964). Bis(triethylammonium) *S*-(4-nitrobenzyl) [$^{17}O_3$]phosphorothioate (1.18 g) was converted to the bis(tri-*n*-octylammonium) salt by stirring a solution of the phosphorothioate in methanol (10 mL) with 2 equiv of tri-*n*-octylamine, until all the tri-*n*-octylamine had been taken up. This was then dried by the addition and evaporation under reduced pressure of dimethylformamide (3 × 8 mL), followed by drying in vacuo for 15 h. It was then dissolved in freshly distilled 1,4-dioxane (8 mL), and freshly distilled diphenyl phosphorochloridate (245 μL) was added to the solution, which was stirred for 3 h. The 1,4-dioxane was evaporated under reduced pressure; the residue was taken up in pyridine (10 mL) and added to a solution of adenosine 5′-[$^{18}O_3$]monophosphate bis(tri-*n*-octylammonium) salt (1.68 mmol) [which had been dried by the addition and evaporation of dry pyridine (3 × 10 mL) in vacuo for 18 h] in pyridine (5 mL). The resulting solution was stirred for 3 h. The solvent was removed under reduced pressure, and the residue was partitioned between 30 mM triethylammonium bicarbonate buffer (50 mL, pH 8.1) and ether (30 mL). The ether was washed with water (50 mL), and the combined aqueous fractions were diluted and applied to a column of DEAE-Sephadex A-25 (150 mL). The column was eluted with triethylammonium bicarbonate buffer (30–400 mM, pH 8.1) at 90 mL h^{-1} over 24 h. The combined product-containing fractions (eluted at 265 mM buffer) were evaporated under reduced pressure, and residual triethylammonium bicarbonate was removed by repeated evaporation of aliquots of methanol (20 mL), to give the product (0.63 g, 797 μmol, 67%). Unreacted adenosine 5′-[$^{18}O_3$]monophosphate (875 μmol) was also recovered from the ion-exchange column. ^{31}P NMR (D_2O): δ 3.6 (P_β), −15.1 (P_α) ($J_{\alpha\beta}$ = 29 Hz); nonbridging ^{18}O shift, δ 0.034; bridging ^{18}O shift, not adequately resolved.

Adenosine 5′-([α,α-$^{18}O_2$,$\alpha\beta$-^{18}O,β,β-$^{17}O_2$]-β-Thiodiphosphate). Adenosine 5′-[*S*-(4-nitrobenzyl) [α-,α-$^{18}O_2$,$\alpha\beta$-^{18}O,β,β-$^{17}O_2$]-β-thiodiphosphate] (780 μmol) was dissolved in methanol (5 mL) and stirred with Dowex-50 (pyridinium form) (40 mL) for 25 min. The solvent was filtered off and evaporated under reduced pressure. The residue was suspended in methanol (10 mL), and tri-*n*-octylamine (700 μL, 2 equiv) was added. The mixture was swirled until all the tri-*n*-octylamine had been taken up. The yellow solution was evaporated under reduced pressure and dried in vacuo for 18 h. Ammonia (60 mL) was condensed at −78 °C under dry nitrogen and dried by distillation from sodium. Excess sodium (ca. 0.4 g) was added to the freshly distilled ammonia, followed immediately (before the sodium dissolved) by the adenosine 5′-[*S*-(4-nitrobenzyl) [α,α-$^{18}O_2$,$\alpha\beta$-^{18}O,β,β-$^{17}O_2$]-β-thiodiphosphate], dissolved in freshly distilled tetrahydrofuran (8 mL). The reaction was stirred until the solution had just started to become blue (about 6 min), at which point the reaction was quenched by the addition of ammonium chloride. The reaction vessel was removed from the cooling bath and the ammonia allowed to evaporate. The residue was dissolved in water and applied to a column of DEAE-Sephadex A-25 (150 mL). The column was eluted with triethylammonium bicarbonate buffer (100–600 mM, pH 8.0) at 90 mL h^{-1} over 24 h. The combined fractions containing the product (eluted at 415 mM buffer) were evaporated under reduced pressure, and residual triethylammonium bicarbonate was removed by repeated evaporation under reduced pressure of aliquots of methanol (20 mL), to give product: 547 μmol, 70% (based on the UV absorbance at 260 nm); ^{31}P NMR (CD_3OD) δ 29.7 (P_β), −14.9 (P_α) ($J_{\alpha\beta}$ = 32 Hz).

(R_P)- and (S_P)-Adenosine 5′-([α,α-$^{18}O_2$,$\alpha\beta$-^{18}O,β-^{17}O,$\beta\gamma$-^{17}O]-β-Thiotriphosphate). This was prepared by the method of Jaffe and Cohn (1978). A solution containing 100 mM HEPES, 100 mM KCl, 12 mM magnesium acetate, 15 mM phosphoenolpyruvate, 0.25 mM EDTA, and 10 mM dithiothreitol was made up and adjusted to pH 7.6 with concentrated sodium hydroxide solution. Adenosine 5′-([α,α-$^{18}O_2$,$\alpha\beta$-^{18}O,β,β-$^{17}O_2$]-β-thiodiphosphate) was dissolved in this solution (54 mL) and incubated with pyruvate kinase (1250 units) at 37 °C. After 24 h, the reaction was complete. The reaction was quenched by the addition of a 100 mM solution of EDTA (15 mL, pH 8.0). The reaction mixture was diluted and applied to a column of DEAE-Sephadex A-25 (120 mL). The column was eluted with triethylammonium bicarbonate buffer (200–800 mM, pH 8.0) at 90 mL h^{-1}. The combined product-containing fractions (eluted at 495 mM buffer) were evaporated under reduced pressure, and residual triethylammonium bicarbonate was removed by repeated evaporation under reduced pressure of aliquots of methanol (20 mL), to give the desired product: 452 μmol, 83%; ^{31}P NMR (CD_3OD) δ 26.6 (P_β), −12.8 (P_γ), −13.8 (P_α) ($J_{\beta\gamma}$ = 28 Hz, $J_{\alpha\beta}$ = 31 Hz).

Adenosine 5′-([(βS)-α,α-$^{18}O_2$,$\alpha\beta$-^{18}O,β-^{17}O,$\beta\gamma$-^{17}O]-β-Thiotriphosphate). The R_P isomer was removed by the procedure of Jaffe and Cohn (1979). A solution containing 40 mM Tris, 2.8 mM magnesium chloride, 40 mM glucose, and 1.4 mM dithiothreitol was adjusted to pH 7.6 with concentrated hydrochloric acid. The mixture of (R_P)- and (S_P)-adenosine 5′-([α,α-$^{18}O_2$,$\alpha\beta$-^{18}O,β-^{17}O,$\beta\gamma$-^{17}O]-β-thiotriphosphate) was dissolved in this solution (300 mL), hexokinase (130 units) was added, and the mixture incubated at 37 °C. When FPLC analysis showed no further change, the reaction was quenched by the addition of 100 mM EDTA (10 mL, pH 8.0). The reaction mixture was diluted and applied to a column of DEAE-Sephadex A-25 (120 mL). The column was eluted with triethylammonium bicarbonate buffer (200–800 mM, pH 8.0) at 90 mL h^{-1}. The combined product-containing fractions (eluted at 495 mM buffer) were evaporated under

reduced pressure, and residual triethylammonium bicarbonate was removed by the repeated evaporation of aliquots of methanol (20 mL). The yield of adenosine 5′-([(βS)-α,α-$^{18}O_2$,αβ-^{18}O,β-^{17}O,βγ-^{17}O]-β-thiotriphosphate) was 357 μmol (79%) and that of adenosine 5′-([α,α-$^{18}O_2$,αβ-^{18}O,β,β-$^{17}O_2$]-β-thiodiphosphate) (which could be recycled) was 79 μmol (17%). The ^{31}P NMR spectrum was identical with that obtained from the mixture of isomers.

Adenosine 5′-([(γR)-α,α-$^{18}O_2$,βγ-^{17}O,γ-^{17}O,γ-^{18}O]-γ-Thiotriphosphate). The title compound was synthesized by the method of Rossomando et al. (1979). A solution was made up containing 40 mM HEPES, 2 mM methionine, 3 mM dithiothreitol, and 5 mM magnesium acetate and adjusted to pH 8.0 with 10 M sodium hydroxide solution. Adenosine 5′-([(βS)-α,α-$^{18}O_2$,αβ-^{18}O,β-^{17}O,βγ-^{17}O]-β-thiotriphosphate) (357 μmol) and bovine serum albumen (18 mg) were dissolved in 180 mL of this solution. The reaction was initiated by the addition of methionyl-tRNA synthetase (230 μL) and was incubated at 30 °C. After 18 h, FPLC analysis showed the reaction to be complete. The reaction was quenched by the addition of 100 mM EDTA (10 mL, pH 8.0). The solution was diluted and applied to a column of DEAE-Sephadex A-25 (120 mL). The column was eluted with a gradient of triethylammonium bicarbonate buffer (150–750 mM, pH 7.9) at 90 mL h^{-1}. The combined product-containing fractions (eluted at 590 mM buffer) were evaporated under reduced pressure, and residual triethylammonium bicarbonate was removed by the repeated evaporation of aliquots of methanol (20 mL), to give the product: 271 μmol, 76%; ^{31}P NMR δ (D_2O) δ 30.0 (d, P_γ), −14.4 (d, P_α), −26.6 (dd, P_β) ($J_{\beta\gamma}$ = 30 Hz, $J_{\alpha\beta}$ = 20 Hz); nonbridging ^{18}O shift (P_γ), δ 0.031; bridging ^{18}O shift (P_γ), δ 0.028.

Incubation of Adenosine 5′-([γ(R)-^{17}O,^{18}O]-γ-Thiotriphosphate) with Glutamine Synthetase. A solution containing 100 mM Tris, 20 mM magnesium chloride, 15 mM dithiothreitol, 50 mM glutamate (monosodium salt), and 100 mM hydroxylamine hydrochloride was prepared and adjusted to pH 7.8. Adenosine 5′-([γ(R)-^{17}O,^{18}O]-γ-thiotriphosphate) (25 μmol) was dissolved in this solution (2.5 mL) and the reaction initiated by the addition of a solution of glutamine synthetase (60 μL). The reaction was incubated at 37 °C for 4.5 h, at which point the reaction was quenched by the addition of 100 mM EDTA (1.5 mL, pH 8.0). The mixture was diluted, adjusted to pH 8.0, and applied to a column of DEAE-Sephadex A-25 (10 mL). The column was eluted with a gradient of triethylammonium bicarbonate buffer (30–300 mM, 750 mL, pH 8.0). The fractions (10 mL) were tested for the presence of inorganic thiophosphate by being spotted onto a silica TLC plate and sprayed with a solution of palladium chloride (1 g in 100 mL of 1 M HCl). Those fractions (eluted at 95 mM buffer) which gave brown spots were combined, evaporated, and desalted by the addition and evaporation of aliquots of methanol (10 mL) to yield inorganic [^{16}O,^{17}O,^{18}O]thiophosphate (9.1 mg), contaminated by a small amount of inorganic phosphate. ^{31}P NMR (CD_3OD): δ 42 ppm (br s).

(S)-S-(1-Phenyl-1-hydroxy-2-ethyl) [^{16}O,^{17}O,^{18}O]-Phosphorothioate. The inorganic [^{16}O,^{17}O,^{18}O]thiophosphate was dissolved in methanol (2 mL) and stirred with Dowex-50 (pyridinium form) (5 mL) for 25 min. The Dowex resin was filtered off and washed with water (3 × 5 mL) and methanol (3 × 5 mL). The solvent was evaporated, and the residue was suspended in methanol (5 mL). Tri-*n*-octylamine (25 μL) was added and the mixture swirled until it had all been taken up. The methanol was evaporated and the inorganic [^{16}O,^{17}O,^{18}O]thiophosphate bis(tri-*n*-octylammonium) salt dried by the evaporation of dry dimethylformamide (3 × 3 mL). The salt was dissolved in dry dimethylformamide (0.5 mL) and (*S*)-(+)-2-iodo-1-phenylethan-1-ol (Arnold et al., 1987) (10 mg) was added. The solution was stirred in the dark for 24 h and then poured into 100 mM triethylammonium bicarbonate buffer (10 mL, pH 9.8). This solution was further diluted and applied to a column of DEAE-Sephadex A-25 (10 mL). The column was eluted with a gradient of triethylammonium bicarbonate buffer (25–250 mM, 750 mL, pH 8.0). Fractions (10 mL) containing the product (eluted at 100 mM buffer and revealed by the palladium chloride spray test) were combined, evaporated, and desalted by the addition and evaporation of aliquots of methanol (10 mL). NMR analysis of the product, using dimethylformamide (3.8 μL) as an internal integration standard, showed that the yield of product was 10.0 μmol, contaminated by inorganic thiophosphate (3.0 μmol) and inorganic phosphate (2.8 μmol): ^{31}P NMR ($C-D_3OD$) δ 12.5.

Cyclization of (S)-S-(1-Phenyl-1-hydroxy-2-ethyl) [^{16}O,^{17}O,^{18}O]Phosphorothioate. A solution of the alkylated thiophosphate in methanol (1 mL) was added to Dowex-50 (pyridinium form) (5 mL) and the mixture stirred for 25 min. The resin was filtered off and washed with water (3 × 5 mL) and methanol (3 × 5 mL). The solvent was evaporated and the residue suspended in methanol (5 mL). Tri-*n*-butylamine (7.5 μL) was added, the mixture stirred for 5 min, and the solvent evaporated. The resulting bis(tri-*n*-butylammonium) salt was dried by the evaporation of dry dimethylformamide (3 × 3 mL) and dissolved in dry dimethylformamide (0.5 mL). To this solution was added tri-*n*-butylamine (3.8 μL) and diphenyl phosphorochloridate (3.2 μL), and the clear solution was stirred for 5 min. The reaction was stopped by the addition of 100 mM triethylammonium bicarbonate buffer (10 mL, pH 9.8). The mixture was diluted and applied to a column of DEAE-Sephadex A-25 (10 mL). The column was eluted with a gradient of triethylammonium bicarbonate buffer (5–120 mM, 750 mL, pH 8.0). Product-containing fractions (10 mL) (eluted at 28 mM buffer) were combined, evaporated, and desalted by the addition and evaporation of aliquots of methanol (10 mL): ^{31}P NMR (CD_3OD) δ 30.2.

Methylation of the Cyclized (S)-S-(1-Phenyl-1-hydroxy-2-ethyl) [^{16}O,^{17}O,^{18}O]Phosphorothioate. A solution of the cyclized phosphorothioate in methanol (1 mL) was added to Dowex-50 (pyridinium form) (5 mL) and stirred for 25 min. The Dowex was filtered off and the resin washed with water (3 × 5 mL) and methanol (3 × 5 mL). The filtrate was evaporated and the resulting pyridinium salt dried by the addition and evaporation of dry acetonitrile (3 × 3 mL). The residue was dissolved in dry acetonitrile (0.5 mL), and to this solution was added a solution of diazomethane in ether (1 mmol in 3 mL, dried by standing over KOH pellets for 1 h). The solution was stirred in the dark for 24 h. The excess diazomethane and the solvent were removed, and the methylated products were analyzed by high-resolution ^{31}P NMR spectroscopy (CD_3CN): δ 41.9 (trans), 40.2 (cis) (see Figure 1).

Results and Discussion

Synthesis of Adenosine 5′-([γ(R)-^{17}O,^{18}O]-γ-Thiotriphosphate). Our method for the synthesis of adenosine 5′-([γ(*R*)-^{17}O,^{18}O]-γ-thiotriphosphate) uses a combined chemical and enzymic strategy. Chemical methods are used to introduce sulfur and the heavy oxygen isotopes, while enzymic methods are used to introduce the chirality. The synthetic strategy combines some features of two published routes to ADPβS

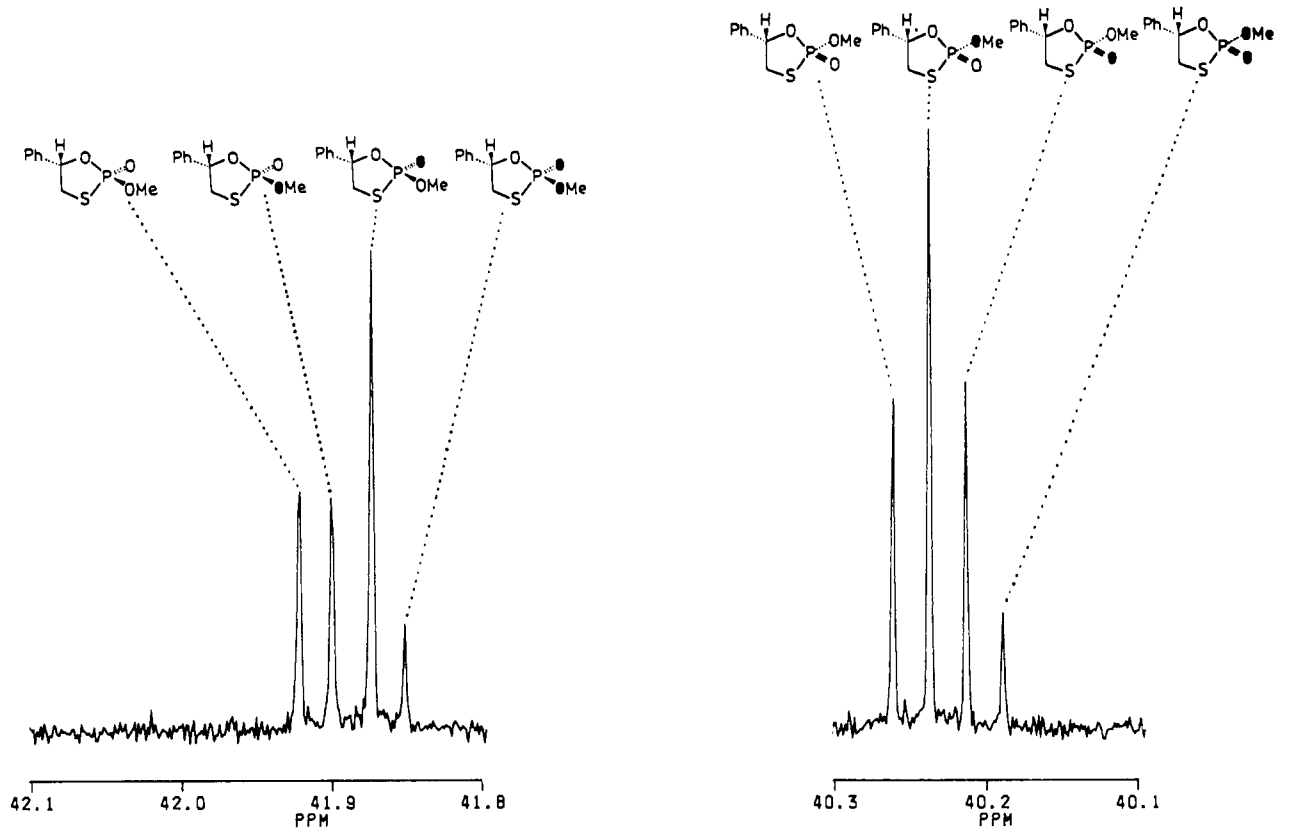
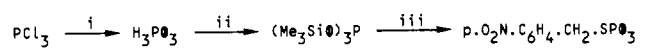


FIGURE 1: ^{31}P NMR spectrum at 202.46 MHz of the labeled *cis*- and *trans*-4(*S*)-phenyl-2-methoxy-2-oxo-1,3,2-thiaoxaphospholanes derived from the inorganic [^{16}O,^{17}O,^{18}O]thiophosphate obtained by incubating adenosine 5′-([γ(*R*)-^{17}O,^{18}O]-γ-thiotriphosphate) with glutamate, hydroxylamine, and glutamine synthetase from *S. typhimurium*. Spectral parameters: sweep width, 4065 Hz; pulse width, 3 μs (49°); acquisition time, 4.0 s; broad-band proton decoupled; Gaussian multiplication, 0.34; line broadening, −0.1 Hz in 32K.

Scheme II: Synthetic Route of *S*-(*p*-Nitrobenzyl) [$^{17}O_3$]Phosphorothioate[a]



[a] * = ^{17}O. Reagents: (i) [^{17}O]water; (ii) Me_3SiCl, Et_3N; (iii) (a) (p-$O_2NC_6H_4CH_2S)_2$, (b) aqueous NaOH.

(Goody & Eckstein, 1971; Ho & Frey, 1984), but our approach was governed by two additional constraints. First, the S-protected [$^{17}O_3$]thiophosphate must be activated in order to introduce the correct labeling pattern at phosphorus, and second, the yield must be optimized with respect to the incorporation of [^{17}O]water.

The method of choice for the synthesis of oxygen-labeled *S*-alkyl phosphorothioates is to produce phosphorous acid by the action of water on phosphorus trichloride, then form its trimethylsilyl triester, and convert this with the appropriate bis(alkyl) disulfide to the product (Bethell & Lowe, 1987). Hydrolysis of the trimethylsilyl ester yields the alkyl phosphorothioate in essentially quantitative yield (although some decomposition of the product occurs during chromatographic purification). In the case of *S*-(*p*-nitrobenzyl) [$^{17}O_3$]-phosphorothioate (Scheme II), a small amount (<5%) of inorganic thiophosphate was produced during hydrolysis. This was readily separated chromatographically from the required product.

Activation of the *S*-(*p*-nitrobenzyl) [$^{17}O_3$]phosphorothioate with diphenyl phosphorochloridate, followed by displacement of diphenyl phosphate by adenosine 5′-[$^{18}O_3$]phosphate (Bethell & Lowe, 1987), completed the introduction of the heavy oxygen isotopes. In the original paper describing this coupling procedure (Michelson, 1964), the activated intermediate is separated from the reaction mixture by precipitation with ether or, in some later modifications, ether and petroleum ether [e.g., Ho and Frey (1984)]. We found precipitation to be inefficient, the yield of labeled *S*-(*p*-nitrobenzyl) ADPβS rising threefold when precipitation was omitted. In order to exclude the possibility that unreacted diphenyl phosphorochloridate might activate the adenosine 5′-[$^{18}O_3$]phosphate and be followed by displacement with unreacted *S*-(*p*-nitrobenzyl) [$^{17}O_3$]phosphorothioate, a preliminary study was conducted in which *S*-benzyl [$^{18}O_3$]phosphorothioate was coupled with unlabeled AMP, using the modified procedure. High-resolution ^{31}P NMR spectroscopic analysis of the product showed that no ^{18}O had been incorporated into the $\alpha\beta$- bridge, proving that the modification does not give rise to alternative labeling in the product (the signal to noise was better than 60 for this spectrum). The *S*-(*p*-nitrobenzyl) group was removed by treatment with sodium in liquid ammonia (Scheme III). ADPβS with the sulfur protected as its benzyl and *o*-nitrobenzyl ester were also synthesized, but their cleavages, reductively and photolytically, respectively, were less efficient.

Pyruvate kinase phosphorylates ADPβS in the presence of phosphoenolpyruvate to give a mixture of S_P and R_P isomers of ATPβS in a ratio of approximately 6:1 (Jaffe & Cohn, 1978). Although phosphoglycerate kinase is known to phosphorylate ADPβS to give exclusively the S_P isomer, it does not proceed to completion (Stegelin et al., 1980; Jaffe et al., 1982). Since (R_P)-ADPβS is exclusively digested by yeast hexokinase in the presence of D-glucose, this reaction was used to obtain the pure S_P diastereoisomer. Since the phosphorylation of ADPβS and dephosphorylation of ATPβS do not effect the isotopic content of ADPβS, the recovered material may be recycled.

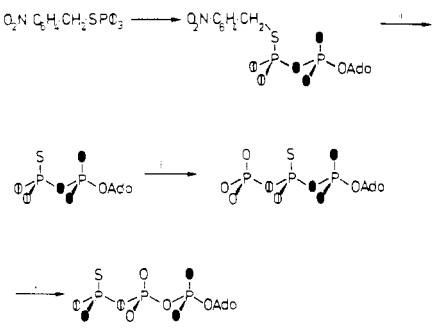
Most aminoacyl-tRNA synthetases will catalyze the formation of their specific aminoacyl adenylate from the amino acid and Mg-ATP, and in the absence of the cognate tRNA

Table I: Observed and Calculated Relative ^{31}P NMR Intensities[a]

	trans triester			cis triester		
		calcd			calcd	
labeled triester	obsd	retention	inversion	obsd	retention	inversion
MeO—P=O	0.53	0.56	0.56	0.55	0.56	0.56
MeO—P=O	0.49	1.00	0.51	1.00	0.51	1.00
MeO—P=●	1.00	0.51	1.00	0.57	1.00	0.51
Me●—P=●	0.23	0.20	0.20	0.20	0.20	0.20

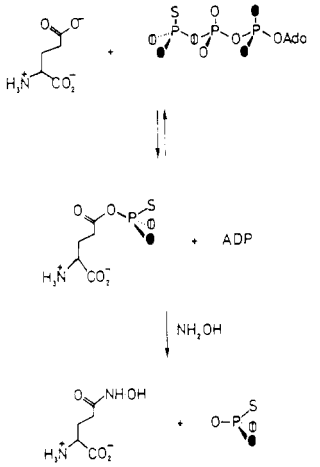
[a] Observed relative signal intensities from the ^{31}P NMR resonances (from Figure 1) of the mixture of trans and cis triesters derived from inorganic [^{16}O,^{17}O,^{18}O]thiophosphate obtained from the glutamine synthetase reaction with adenosine 5′-([$\gamma(R)$-^{17}O,^{18}O]-γ-thiotriphosphate). The calculated values were derived by a computer program to give the best fit and incorporate a 9% loss of isotope with retention.

Scheme III: Synthetic Route to Adenosine 5′-([(γR)-α,α-$^{18}O_2$,$\beta\gamma$-^{17}O,γ-^{17}O,γ-^{18}O]-γ-Thiotriphosphate)[a]



[a] Φ = ^{17}O; ● = ^{18}O. Reagents: (i) (a) $(PhO)_2POCl$, (b) [$^{18}O_3$]-AMP; (ii) Na, liquid NH_3; (iii) (a) pyruvate kinase, phosphoenolpyruvate, (b) hexokinase, D-glucose; (iv) methionyl-tRNA synthetase, methionine.

Scheme IV: Stereochemical Course of the Activation of Glutamate by Glutamine Synthetase



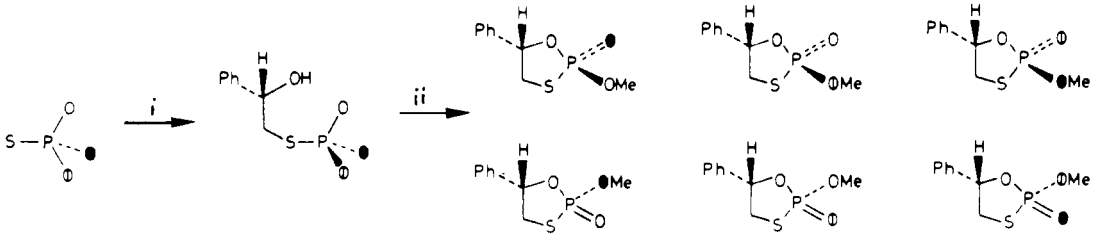
and inorganic pyrophosphatase, the reaction is reversible (Schimmel & Soll, 1979). Methionyl-tRNA synthetase accepts (S_P)-ATPβS as a substrate and, in the presence of methionine, catalyzes its conversion to ATPγS, the thermodynamically more stable nucleotide (Rossomando et al., 1979). This enzyme-catalyzed rearrangement enables the adenosine 5′-([(βS)-α,α-$^{18}O_2$,$\alpha\beta$-^{18}O,β-^{17}O,$\beta\gamma$-^{17}O]-β-thiotriphosphate) to be converted to the target molecule (Scheme III). By recycling ADPβS recovered from the hexokinase digestion, the overall yield of product was 320 μmol (16% from PCl_3).

Stereochemical Course of the Activation of Glutamate by Glutamine Synthetase. A wealth of evidence has been presented to support the intermediacy of γ-glutamyl phosphate in the mechanism of glutamine synthetase. This has come from pulse–chase experiments, studies with substrate analogues and transition-state analogues [summarized in Meister (1974)], trapping experiments (Todhunter & Purich, 1975), positional isotope exchange, and kinetic studies, which demonstrated its chemical and kinetic competence (Midelfort & Rose, 1976; Meek et al., 1982).

The use of chiral phosphates and chiral thiophosphates has, over the past decade, provided much valuable information about the chemical mechanisms of a large number of enzymes (Lowe, 1983; Eckstein, 1985). Ideally the phosphate residue is made chiral by isotopic substitution alone, but when the product is inorganic phosphate, it is not possible, since only three stable isotopes of oxygen exist. In such cases the phosphorus must be made chiral by the substitution of oxygen by sulfur. Such thiophosphate analogues have been widely used for the study of enzyme-catalyzed phosphoryl transfer reactions (Eckstein, 1985). Although they tend to be poor substrates for enzymes, all enzymes that have been studied with chiral phosphates and the corresponding chiral thiophosphate analogues have been shown to follow the same stereochemical course (Eckstein, 1985).

In a preliminary experiment ATPγS was shown to be quite a good substrate for the unadenylated glutamine synthetase from *Salmonella typhimurium*, its turnover being approximately $^1/_{20}$ of that of ATP. Adenosine 5′-([$\gamma(R)$-^{17}O,^{18}O]-γ-thiotriphosphate) was incubated with glutamine synthetase in the presence of hydroxylamine (to cleave the γ-glutamyl [^{17}O,^{18}O]thiophosphate) and the chiral [^{16}O,^{17}O,^{18}O]thiophosphate isolated by ion-exchange chromatography (Scheme IV). The configuration of the chiral [^{16}O,^{17}O,^{18}O]thiophosphate was determined by the newly developed method of

Scheme V: Reaction Scheme for Analysis of Chiral [^{16}O,^{17}O,^{18}O]Thiophosphate Generated from the Activation of Glutamate by Glutamine Synthetase with Adenosine 5′-([$\gamma(R)$-^{17}O,^{18}O]-γ-Thiotriphosphate) As Depicted in Scheme IV[a]



[a] Reagents: (i) (*S*)-2-iodo-1-phenylethanol; (ii) (a) $(PhO)_2POCl$, *n*-Bu_3N, (b) CH_2N_2.

stereochemical analysis (Arnold & Lowe, 1986; Arnold et al., 1987). The chiral [^{16}O,^{17}O,^{18}O]thiophosphate was treated with (*S*)-2-iodo-1-phenylethanol, and the (*S*)-*S*-(1-phenyl-1-hydroxy-2-ethyl) [^{16}O,^{17}O,^{18}O]thiophosphate formed was cyclized with diphenyl phosphorochloridate. The cyclization proceeds with inversion of configuration at phosphorus (Arnold & Lowe, 1986; Arnold et al., 1987). After isolation by ion-exchange chromatography, the isotopomers of the cyclic thiophosphate were methylated with diazomethane to give a mixture of the *trans*- and *cis*-methyl esters (Scheme V). The ^{31}P NMR spectrum of the isotopomeric mixture of *trans*- and *cis*-methyl esters is shown in Figure 1. The spectrum reveals only those isotopomers containing ^{16}O and ^{18}O, since ^{17}O directly bonded to phosphorus causes broadening of the ^{31}P resonance by scalar relaxation (Lowe et al., 1979; Tsai, 1979; Tsai et al., 1980). When ^{18}O is directly bonded to phosphorus, an isotope shift to higher field is observed (Cohn & Hu, 1978; Lowe & Sproat, 1978), the magnitude of which is related to the bond order. When ^{18}O is in a phosphorus–oxygen double bond, the isotope shift is approximately twice that in a phosphorus–oxygen single bond (Lowe et al., 1979). The three lowest intensity resonances in each quartet of isotopomers arise because the ^{17}O site is not fully enriched. Since the predominant isotopomer has ^{18}O in the P=O bond for the trans-isomer and in the P–OMe bond for the cis- isomer, the chiral [^{16}O,^{17}O,^{18}O]thiophosphate has the *S* configuration (as depicted in Schemes IV and V), and hence, the reaction has proceeded with inversion of configuration at phosphorus. The comparison of the observed relative peak intensities (from Figure 1) with those calculated (Table I) clearly shows that the reaction proceeds with complete inversion of configuration (within experimental limits). Alone this proves that the reaction involves an odd number of "in-line" displacements at phosphorus. Midelfort and Rose (1976), however, showed that positional isotope exchange from the $\beta\gamma$-bridging position in ATP to the β-nonbridging position occurred in the presence of glutamate and enzyme, but not with enzyme alone or with methionine sulfoximine (a potent inhibitor, which induces a conformational change in the enzyme similar to that of glutamate) and enzyme. Taken together the two experiments show that the mechanism of activation of glutamate by glutamine synthetase involves a single, in-line, phosphoryl transfer step to give the intermediate γ-glutamyl phosphate.

ACKNOWLEDGMENTS

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Registry No. EC 6.3.1.2, 9023-70-5; Glu, 56-86-0; $O_2NC_6H_4CH_2SP^{17}O_3 \cdot 2Et_3N$, 112221-01-9; $O_2NC_6H_4CH_2SP^{17}O_3 \cdot 2[H_3C(CH_2)_7]N$, 112221-02-0; [$^{18}O_3$]AMP, 108275-88-3; Ado 5′-[*S*-(4-nitrobenzyl)[α-$^{18}O_2$,$\alpha\beta$-^{18}O,β-$^{17}O_2$]-β-thiodiphosphate], 108275-89-4; Ado 5′-([α-$^{18}O_2$,$\alpha\beta$-^{18}O,β-$^{17}O_2$]-β-thiodiphosphate), 108275-90-7; (R_P)-Ado 5′-([α-$^{18}O_2$,$\alpha\beta$-^{18}O,β-^{17}O,$\beta\gamma$-^{17}O]-β-thiotriphosphate), 112295-48-4; (S_P)-Ado 5′-([α-$^{18}O_2$,$\alpha\beta$-^{18}O,β-^{17}O,$\beta\gamma$-^{17}O]-β-thiotriphosphate), 112295-49-5; Ado 5′-([α-$^{18}O_2$,$\beta\gamma$-^{17}O,γ-^{18}O,γ-^{17}O]-γ-thiotriphosphate), 108275-92-9.

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