Synthesis and Characterization of Diastereomers of Guanosine 5′-*O*-(1-Thiotriphosphate) and Guanosine 5′-*O*-(2-Thiotriphosphate)†

Bernard A. Connolly, Paul J. Romaniuk, and Fritz Eckstein*

ABSTRACT: The synthesis and characterization of guanosine 5′-*O*-(1-thiotriphosphate) (GTPαS) and guanosine 5′-*O*-(2-thiotriphosphate) (GTPβS) using chemical and enzymatic methods are described. GTPαS A (S_P diastereomer) can be prepared enzymatically from a chemically synthesized mixture of the diastereomers of guanosine 5′-*O*-(1-thiodiphosphate) (GDPαS) with phosphoglycerate kinase. GTPαS B (R_P diastereomer) can be similarly synthesized with succinyl-CoA synthetase and by back-digesting the small amounts of GTPαS A formed with phosphoglycerate kinase. Guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS) serves as the precursor for both GTPβS A (S_P diastereomer), prepared with pyruvate kinase and by back-digesting with glycerol kinase, and GTPβS B (R_P diastereomer), obtained with acetate kinase and by back-digesting with myosin. These analogues can be γ-^{32}P labeled by $^{32}P_i$ exchange with either phosphoglycerate kinase–phosphoglyceraldehyde dehydrogenase or succinyl-CoA synthetase. Finally, the interaction of these four nucleotides with acetate kinase, RNA polymerase, and succinyl-CoA synthetase is described.

Since their introduction, the phosphorothioate analogues of ATP, in which a nonbridging oxygen atom on the α, β, or γ phosphorus is replaced by a sulfur, have been extensively used to study ATP-dependent enzymes [for reviews, see Eckstein (1978, 1980) and Eckstein et al. (1982)]. Such a substitution at the α or β phosphorus introduces an additional center of chirality into the nucleotide so that both the α- and β-phosphorothioates exist as pairs of diastereomers for which the absolute configurations are known (Burgers & Eckstein, 1978; Burgers et al., 1979; Jaffe & Cohn, 1978a, 1979; Bryant & Benkovic, 1979). At their simplest these analogues can be used as probes for the stereoselectivity of an enzyme nucleotide binding site. Another application is in the determination of the structure of the metal–nucleotide chelate active with a particular enzyme. This method relies on the preferential coordination of Mg^{2+} to oxygen and Cd^{2+} to sulfur in the phosphorothioates and the consequent generation of specific metal–nucleotide chelates rather than the rapidly equilibrating mixture of chelates formed with Mg^{2+} and ATP (Jaffe & Cohn, 1978a, 1979). Finally, the phosphorothioates, often in conjunction with the stable isotopes of oxygen, have been used to elucidate the stereochemical course of phosphoryl and adenylyl transfer reactions. Knowing whether these groups are transferred with inversion or retention of configuration greatly restricts the number of mechanisms that can be proposed for an enzyme and also provides evidence for or against a covalent enzyme substituted intermediate [see above reviews and Knowles (1980)].

Like adenosine, guanosine and its mono-, di-, and triphosphate derivatives are ubiquitous components of living cells. Many enzymes normally considered ATP dependent will also utilize GTP albeit at usually lower rates. Additionally, a few enzymes, e.g., mammalian succinyl-CoA synthetase (Sandai et al., 1955; Cha, 1969), phosphoenolpyruvate carboxykinase (Chang et al., 1966), and guanylate cyclase (Goldberg & Haddox, 1977; Macchia et al., 1975), are specific for GTP. Between these two extremes lie the DNA and RNA polymerases for which ATP and GTP must be considered cosubstrates. Perhaps more importantly, guanosine nucleotides are critically involved in a number of complex and important biological processes. Thus protein synthesis is dependent on initiation and elongation factors, soluble proteins that bind GTP and possess GTPase activities (Weissbach & Ochoa, 1976). Similarly, the polymerization of tubulin into microtubules seems to require both GTP binding and hydrolysis (Timasheff & Grisham, 1980). Extensive studies have shown that GTP is also involved in the hormone-mediated activity of adenylate cyclase. In this case GTP is bound and hydrolyzed by a GTP binding regulatory protein in a hormone-sensitive manner [for reviews, see Ross & Gilman (1980), Rodbell (1980), and Limbird (1981)].

In the hope of extending the phosphorothioate method to the above processes, this paper describes the synthesis of the α- and β-phosphorothioate analogues of GTP. Facile enzymatic methods for introducing γ-^{32}P into these nucleotides are also reported. Finally, the interaction of these GTP derivatives with a number of enzymes is discussed.

Materials and Methods

ATP and GTP were obtained from Pharma Waldhof (Mannheim, Germany). GDPβS[1] was purchased from Boehringer Mannheim (Germany) and routinely found to be contaminated with up to 30% GMP and GDP. Purification could be achieved by ion-exchange chromatography on DEAE-Sephadex A-25 and gradient elution with 0.1–0.5 M TEAB. However, the GDPβS was normally used for triphosphate synthesis as supplied as these impurities were removed during the purification of the GTPβS product. Calf thymus DNA was the product of Boehringer Mannheim (Germany). DL-Glyceraldehyde 3-phosphate diethyl acetal

† From the Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, D-3400 Göttingen, West Germany. *Received July 13, 1981.*

[1] Abbreviations: GMPS, guanosine 5′-phosphorothioate; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); GDPαS A and B, the S_P and R_P diastereomers of guanosine 5′-*O*-(1-thiodiphosphate), respectively; GTPαS A and B, the S_P and R_P diastereomers of guanosine 5′-*O*-(1-thiotriphosphate), respectively; GTPβS A and B, the S_P and R_P diastereomers of guanosine 5′-*O*-(2-thiotriphosphate), respectively; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); the adenosine phosphorothioate analogues are abbreviated in an exactly similar fashion; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; TEAB, triethylammonium bicarbonate; DEAE, diethylaminoethyl; CoA, coenzyme A; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; NAD, nicotinamide adenine dinucleotide.

0006-2960/82/0421-1983$01.25/0

was obtained from Boehringer Mannheim and hydrolyzed to the active aldehyde in accordance with the manufacturer's instructions. The glyceraldehyde 3-phosphate so formed was stored frozen at pH ~2 and used within 1 week of preparation. Succinyl-CoA was synthesized from CoA (Boehringer Mannheim) and succinic anhydride by the procedure of Simon (1957). The succinyl-CoA product was lyophilized, stored at −20 °C, and used without further purification. The following enzymes were obtained from Boehringer Mannheim: acetate kinase (*Escherichia coli*); gluconate kinase (*E. coli*); glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle); glycerol kinase (candida mycoderma); guanosine-5′-monophosphate kinase (porcine brain); hexokinase (yeast); nucleoside-5′-diphosphate kinase (beef liver); nucleoside-5′-monophosphate kinase (beef liver); phosphofructokinase (rabbit muscle); phosphoglycerate kinase (yeast); phosphotransacetylase (*Clostridium kluyveri*); pyruvate kinase (rabbit muscle); succinyl-CoA synthetase (pig heart). *E. coli* DNA-dependent RNA polymerase was a generous gift from Dr. H. Sternbach of this department, and rabbit skeletal muscle myosin was prepared as described (Wiedner et al., 1978). All radiochemicals were purchased from the Radiochemical Centre (Amersham, United Kingdom). Thiophosphoryl chloride was the product of Merck (Darmstadt, West Germany) and was redistilled before use. When indicated, solvents were dried by storage over 4-Å molecular sieves for 45 h.

^{31}P NMR spectra were recorded as described (Eckstein & Goody, 1976), and chemical shifts are given in ppm downfield from 85% aqueous phosphoric acid as the external standard. Guanosine nucleotide concentrations were measured, at pH 7, with a Zeiss PMQ II spectrophotometer and an E_{252}^{1mM} = 13.7.

All HPLC experiments were performed with a Waters Associates (Model 6000 A) liquid chromatograph fitted with a 254-nm absorbance detector (Model 440). Reactions were routinely monitored with a 20 × 0.4 cm column packed with the anion exchanger Nucleosil 10 SB (Machery-Nagel, Düren, West Germany) and eluted isocratically with 50 mM KH_2PO_4, pH 4.5, containing 500 mM KCl. This system resolved mono-, di-, and triphosphates and also distinguished between normal nucleotides and their phosphorothioate analogues. When the synthesis of GTPαS A or B from a mixture of the diastereomers of GDPαS was studied, 50 mM KH_2PO_4, pH 6.9, containing 400 mM KCl was used as the eluent. This system separated GDPαS A, GDPαS B, GTPαS A, and GTPαS B, which eluted in that order. A reverse-phase system, consisting of the C_{18} derivative ODS-Hypersil (Shandon Southern Products Ltd., Runcorn, United Kingdom) eluted isocratically with 50 mM KH_2PO_4, pH 6 (P. A. Frey, personal communication), was used to confirm the purity of the products. This system gave the best resolution of the diastereomers of GDPαS and GTPαS but could not be used routinely to monitor reactions as the nucleotides tended to coelute with UV-absorbing impurities present in the buffers. None of the HPLC systems resolved the diastereomers of GTPβS. When ascertaining the purity of γ-^{32}P-labeled nucleotides, we collected 0.5-mL fractions and determined the amount of radioactivity present by scintillation counting in water with the ^{3}H channel.

Chemical Syntheses. (*1*) *Guanosine 5′-Phosphorothioate.* Guanosine (2.8 g; 10 mmol) was dried overnight in vacuo over P_2O_5 at 80 °C and then suspended in 50 mL of dry trimethyl phosphate. After the mixture was stirred at room temperature for 1 h, during which time most of the guanosine dissolved, 2.1 mL (20 mmol) of redistilled thiophosphoryl chloride was added and the mixture stirred for a further 3 h. Complete solution of the guanosine occurred within 15 min. After 3 h, 100 mL of 10% aqueous barium acetate solution was added, followed 15 min later by 400 mL of ice-cold ethanol containing 10 mL of triethylamine. After a further hour at 0 °C, the dense white suspension was centrifuged and the supernatant discarded. The precipitate was extracted 3 times with 100 mL of an ice-cold ethanol–water (7:3 v/v) mixture with the supernatant being discarded each time. The precipitate was then extracted with 6 × 100 mL of water, and the combined supernatants were pooled and freed from barium by passage over a 60 × 3 cm Merck I ion-exchange resin (pyridinium form). The eluate and washings from this column were adjusted to pH 8 with triethylamine and concentrated to ~30 mL by rotary evaporation at 35 °C. The crude product was purified by chromatography on a column (45 × 3 cm) of DEAE-Sephadex A-25 equilibrated to 0.05 M TEAB. After washing with 1 L of this buffer, we developed the column with a linear gradient of 1.75 L each of starting buffer and 0.4 M buffer. Fractions containing product (which eluted at about 0.35 M TEAB) were pooled and dried by rotary evaporation. Excess buffer was removed by repeated coevaporation with methanol. The product was finally dissolved in 25 mL of water, adjusted to pH 7, and stored frozen at −20 °C. Yields were typically 35% and the product appeared 95% pure in both the anion-exchange and reverse-phase HPLC system. ^{31}P NMR showed one major peak at 42.87 ppm, and a typical guanosine nucleotide UV spectrum was obtained.

(*2*) *Guanosine 5′-O-(1-Thiodiphosphate).* This was prepared by the activation of guanosine 5′-phosphorothioate with diphenyl phosphochloridate and subsequent addition of phosphate exactly as given by Eckstein & Goody (1976) for adenosine 5′-*O*-(1-thiodiphosphate). The reaction was usually performed with 2.5 mmol of GMPS and appropriate scaleup in conditions. Purification was by chromatography over a 50 × 4 cm column of DEAE-Sephadex A-25, which was washed with 2 L of 0.1 M TEAB and then eluted with a gradient composed of 1.75 L each of 0.1 and 0.65 M TEAB. The product, which eluted at about 0.55 M TEAB, was obtained in 10% yield. Two major peaks of equivalent area, representing the diastereomers of GDPαS, were seen on reverse-phase HPLC. ^{31}P NMR showed two doublets at 40.65 (α-P) and −6.94 (β-P) ppm.

Enzymatic Syntheses. All enzymatic syntheses were performed at room temperature and monitored by anion-exchange HPLC. Product purification was by chromatography on DEAE-Sephadex A-25 (25 × 3 cm columns) equilibrated with 0.1 M TEAB, pH 7.5, and eluted with a gradient of 1.25 L each of 0.1 and 0.65 M TEAB. The triphosphate products, all of which eluted at about 0.6 M buffer, were dried down as outlined above.

Guanosine 5′-O-(1-Thiotriphosphate), Isomer A. (1) To an incubation mixture (total volume 25 mL) containing 50 mM KH_2PO_4, pH 7, 16 mM glyceraldehyde phosphate, 10 mM $MgCl_2$, 1 mM EDTA, 3.6 mM GDPαS (50:50 mixture of the diastereomers obtained above), and 1.8 mM NAD were added 900 units of phosphoglycerate kinase and 160 units of phosphoglyceraldehyde dehydrogenase. The reaction was complete in 30 min, and purification by chromatography on DEAE-Sephadex A-25 yielded 22 μmol of product consisting of 95% GTPαS A and 5% GTPαS B.

(2) To a 5.0-mL incubation mixture containing 100 mM Tris, pH 7.5, 5 mM $MgCl_2$, 2 mM GDPαS A (containing 5% B isomer; prepared by the chromatographic separation described below), 1 mM ATP, 1 mM phosphoenolpyruvate, and 1 mM dithioerythritol were added 10 units of pyruvate kinase

and 200 units of nucleoside diphosphokinase. After 2 h at room temperature, the reaction mixture was purified by DEAE-Sephadex A-25 column chromatography to yield 5 μmol of 100% GTPαS A.

Guanosine 5'-O-(1-Thiotriphosphate), Isomer B. (1) To a 25-mL incubation mixture containing 100 mM Hepes, pH 7.5, 50 mM KH_2PO_4, 10 mM $MgCl_2$, 25 mM acetyl phosphate, 3.6 mM GDPαS (mixture of diastereomers), and 1.8 mM succinyl-CoA were added 15 units of succinyl-CoA synthetase and 100 units of phosphotransacetylase. After 30 min the reaction mixture was worked up, yielding 41 μmol of GTPαS consisting of 85% GTPαS B and 15% GTPαS A. Further purification was achieved by converting the GTPαS A to GDPαS A with phosphoglycerate kinase. The reaction mixture contained, in 25 mL, 100 mM triethanolamine, pH 8, 20 mM semicarbazide, 10 mM $MgCl_2$, 1 mM EDTA, 2 mM NaOH, 1.3 mM 3-phosphoglycerate, and the 41 μmol (final concentration 1.64 mM) of triphosphate obtained above. The reaction was started by adding 1000 units of phosphoglycerate kinase and 100 units of phosphoglyceraldehyde dehydrogenase, and after 2 h, purification yielded 30 μmol of product consisting of 95% GTPαS B and 5% GTPαS A.

(2) To a 5-mL incubation mixture containing 100 mM Tris, pH 7.4, 5.0 mM $MgCl_2$, 2.2 mM GDPαS B (containing less than 1% of the A isomer obtained chromatographically as described below), 2 mM dithioerythritol, and 20 mM acetyl phosphate was added 10 units of acetate kinase, and the reaction was incubated 2 h at room temperature. Purification by column chromatography yielded 7 μmol of 99% GTPαS B isomer.

Guanosine 5'-O-(2-Thiotriphosphate), Isomer A. To an incubation mixture (total volume 10 mL) containing 100 mM Tris, pH 7.5, 200 mM KCl, 25 mM $MgCl_2$, 50 mM phosphoenolpyruvate, 1 mM dithiothreitol, and 10 mM GDPβS was added 2000 units of pyruvate kinase. After 4 h, chromatography yielded 70 μmol of product consisting of a 70:30 mixture of GTPβS A and GTPβS B. Pure GTPβS A was prepared by removal of GTPβS B with glycerol kinase. The reaction mixture contained, in 15 mL, 100 mM glycine–KOH, pH 9.8, 100 mM glycerol, 10 mM $MgCl_2$, 1 mM dithiothreitol, and the 70 μmol of triphosphate produced above (final concentration 4.7 mM). Glycerol kinase (150 units) was added, followed 3 h later by a further 60 units. After a further 2 h, chromatography yielded 45 μmol of 100% GTPβS A.

Guanosine 5'-O-(2-Thiodiphosphate), Isomer B. To a 10-mL incubation mixture containing 100 mM Tris, pH 7.5, 10 mM $MgCl_2$, 50 mM acetyl phosphate, 1 mM dithiothreitol, and 10 mM GDPβS was added 200 units of acetate kinase. After 90 min, purification by column chromatography yielded 71 μmol of GTPβS B containing traces of GTP (introduced from GDP contamination of the GDPβS). Pure GTPβS B was obtained by incubating this 71 μmol of product in a volume of 15 mL containing 100 mM Tris, pH 7.5, 400 mM KCl, 7 mM $CaCl_2$, 1 mM dithiothreitol, and 15 mg of myosin. After 3 h the mixture was worked up to give 45 μmol of 100% pure GTPβS B.

Aqueous solutions at pH 7 of the nucleotides were stored at −20 °C after rapid freezing in dry ice–acetone. Less than 5% decomposition in 3 months was observed.

Radiochemical Syntheses. (*1*) *[γ-^{32}P]GTPαS A or B.* These were prepared by equilibrating purified GTPαS A or B with $^{32}P_i$ by using phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase as for the adenosine analogues (Romaniuk & Eckstein, 1981). Typically, a specific activity of 100 μCi/μmol was obtained.

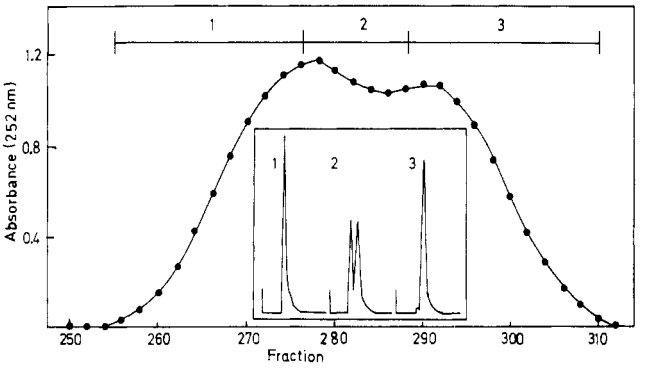

FIGURE 1: Resolution of 20 μmol each of GTPαS A and B by chromatography on DEAE-Sephadex A-25. Fractions 1–3 were pooled as indicated and analyzed by reverse-phase HPLC (insert). Fraction 1 consists of 13 μmol of 95% GTPαS A (retention time 1.7 min), fraction 2 of 11 μmol of 50% GTPαS A and 50% GTPαS B, and fraction 3 of 11 μmol of 95% GTPαS B (retention time 2.3 min).

(*2*) *[γ-^{32}P]GTPβS A or B.* These were prepared from GDPβS and $^{32}P_i$ by using succinyl-CoA synthetase. The reaction mixture contained in a 10-mL total volume 100 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 2 mM GDPβS, 0.5 mM KH_2PO_4 (containing 5 mCi of $^{32}P_i$), 2 mM acetyl phosphate, and 2.5 mM succinyl-CoA. The reaction was started by the addition of 25 units of succinyl-CoA synthetase and 100 units of phosphotransacetylase, and after 1 h at room temperature the triphosphate product was purified by DEAE-Sephadex A-25 chromatography as above. GTPβS (7 μmol), consisting of approximately 40% A diastereomer and 60% B diastereomer, of specific activity 75 μCi/μmol was obtained. Pure [γ-^{32}P]GTPβS A was obtained by removal of GTPβS B with glycerol kinase. The reaction was performed in a 10-mL total volume containing 100 mM glycine–KOH, pH 9.8, 10 mM $MgCl_2$, and 100 mM glycerol containing 4.2 μmol of the GTPβS A and B mixture obtained above; 100 units of glycerol kinase was added, followed by a further 100 units after 2 and 3 h, respectively. After 4 h 2.5 μmol of pure unlabeled GTPβS A was added to aid in UV detection and the mixture purified as above. GTPβS A (3.8 μmol) of specific activity 20 μCi/μmol containing 5% GTPβS B was obtained. Pure [γ-^{32}P]-GTPβS B was obtained by removal of contaminating GTPβS A with myosin. The reaction mixture contained (total volume 10 mL) 100 mM Tris, pH 7.5, 400 mM KCl, 10 mM $CaCl_2$, and 2.8 μmol of the GTPβS A and B mixture synthesized above. Myosin (10 mg) was added, followed by a further 10 mg after 2 h. After a further 2 h 2.5 μmol of pure unlabeled GTPβS B was added and the mixture purified as usual. GTPβS B (3.6 μmol) with a specific activity of 17 μCi/μmol containing 5% GTPβS A was obtained.

Chromatographic Separation of GTPαS A and B. A 50:50 mixture of GTPαS A and B (40 μmol) was applied to a 100 × 2.5 cm column of DEAE-Sephadex A-25 equilibrated to 0.4 M TEAB. Elution was with a linear gradient composed of 2 L each of 0.4 and 0.65 M TEAB. The elution profile is shown in Figure 1 as is the reverse-phase HPLC analysis, which shows that fraction 1 consists of 95% GTPαS A (13 μmol), fraction 2 an equal mixture of the two diastereomers (11 μmol), and fraction 3 95% GTPαS B (11 μmol). This procedure can be used to separate a diastereomeric mixture of GDPαS (by using a gradient from 0.3 to 0.55 M) or to obtain 100% pure samples of GTPαS A or B from the 95% pure preparations obtained enzymatically.

Analysis of Diastereomeric Purity of GTPβS A and B. (*1*) *Glycerol Kinase.* To an incubation mixture (volume 1 mL) containing 100 mM glycine–KOH, pH 9.8, 25 mM glycerol,

2.5 mM $MgCl_2$, and 1 mM GTPβS A or B was added 15 units of glycerol kinase. After 1 h a 25-μL aliquot was analyzed by the anion-exchange HPLC system, which resolved the triphosphate substrate from any GDPβS product.

(*2*) *Myosin.* To an incubation mixture (volume 1 mL) containing 100 mM Tris, pH 7.5, 400 mM KCl, 5 mM $CaCl_2$, 1 mM dithiothreitol, and 1 mM GTPβS A or B was added 5 mg of myosin. After 1 h the reaction was analyzed by HPLC as above.

Enzyme Assays. (*1*) *Acetate Kinase.* This enzyme was assayed by $^{32}P_i$ released from γ-^{32}P-labeled nucleotide (Romaniuk & Eckstein, 1981). Activity was measured at 37 °C in a 1.2-mL total volume containing 100 mM Tris, pH 7.4, 100 mM potassium acetate, 2 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 100 mM hydroxylamine, and nucleotide levels of between 30 and 600 μM. The reaction was initiated by the addition of 3 μg of acetate kinase and $^{32}P_i$ release measured as described (Yee et al., 1980).

(*2*) *RNA Polymerase.* Activity was determined by copolymerization of [^{14}C]CTP onto a calf thymus DNA template at 37 °C. The assay mixture contained, in 200 μL, 100 mM Tris, pH 8, 200 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 1 mM each of ATP, UTP, and [^{14}C]CTP (specific activity approximately 2 μCi/nmol), and 20 μg of calf thymus DNA. The concentration of GTP analogues was varied between 25 and 800 μM and the reaction initiated by addition of 44 μg of RNA polymerase. Aliquots were withdrawn at 3-, 6-, and 9-min intervals, and the amount of [^{14}C]CTP incorporated into polymer was measured as described (Armstrong et al., 1979).

(*3*) *Succinyl-CoA Synthetase.* Activity was measured by recording the increase in optical density at 235 nm resulting from thioester bond formation (Cha, 1969). The assay was performed at 25 °C and contained in a volume of 1 mL 50 mM Hepes and 50 mM succinate, each adjusted to pH 7.4, 10 mM $MgCl_2$, 200 μM CoA, and nucleotide levels of between 5 and 250 μM. The reaction was initiated by the addition of between 2.5 and 50 μg of enzyme, and the initial rates were determined from the slopes of the traces obtained with an $E_{235}^{1mM} = 4$.

The kinetic constants for acetate kinase and RNA polymerase were determined from plots of velocity vs. velocity/substrate concentration, whereas those for succinyl-CoA synthetase were evaluated by plotting substrate concentration/velocity against velocity.

Results and Discussion

The methods given here for synthesis of the diastereomers of GTPαS and GTPβS (Figure 2) are based on those used for the corresponding adenosine analogues (Eckstein & Goody, 1976; Yee et al., 1979; Sheu & Frey, 1977; Midelfort & Sarton-Miller, 1978). However, the change in purine base has entailed several important changes in the procedures followed.

Adenosine 5′-phosphorothioate is generally prepared by the reaction of adenosine with thiophosphoryl chloride in triethyl phosphate (Murray & Atkinson, 1968), a method based on that for the phosphorylation of nucleosides (Yoshikawa et al., 1967). Guanosine was found to be insoluble in this solvent, and product yields varied between 0 and 15%. The use of trimethyl phosphate, in which guanosine is fairly soluble, increased yields to 35%. Additional purification of the GMPS by ion-exchange chromatography was also found to be necessary, whereas the adenosine analogue is essentially pure after the solvent-extraction procedures (Eckstein et al., 1975).

The chemical synthesis of the diastereomers of GDPαS from GMPS proceeded in only 10% yield in contrast to ADPαS,

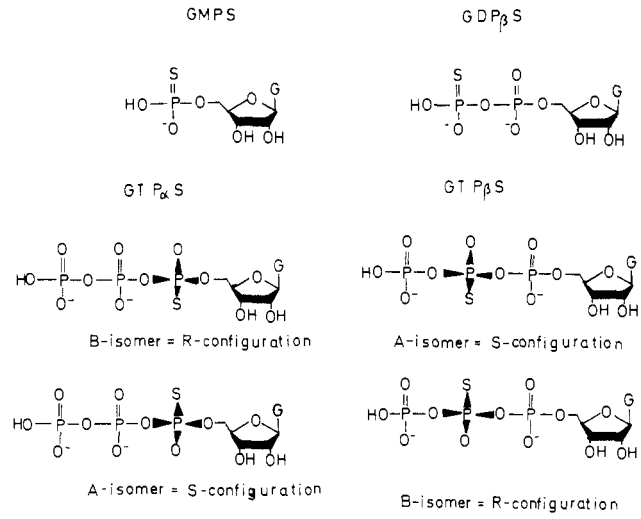

FIGURE 2: G = guaninyl.

which can be prepared in up to 40% yields by an identical method. All attempts to prepare GDPαS from GMPS enzymatically were unsuccessful. With ATP and GMPS as the phosphoryl donor and acceptor, respectively, no reaction was observed with adenylate kinase, guanylate kinase, or nucleoside monophosphate kinase. This contrasts with the facile and stereospecific conversion of AMPS and ADPαS A with adenylate kinase (Sheu & Frey, 1977; Jaffe & Cohn, 1978b).

No enzymes were found that converted the chemically synthesized mixture of GDPαS A and B to one of the triphosphate diastereomers with 100% stereoselectivity. GTPαS A can be prepared with 5% GTPαS B contamination by using phosphoglycerate kinase, providing the second substrate 1,3-diphosphoglycerate is limited to 50% of the total GDPαS concentration (i.e., equal to GDPαS A levels). This compound is generated in situ from 3-phosphoglyceraldehyde by using phosphoglyceraldehyde dehydrogenase, and its level is most conveniently controlled by limiting NAD. The use of an excess of NAD results in a triphosphate product much less enriched in the A diastereomer. Nucleosidediphosphate kinase has been reported to stereoselectively transfer the γ-phosphate of ATP to ADPαS A, resulting in ATPαS A (Eckstein & Goody, 1976). Indeed, this method has been used to resolve GDPαS A and B as well as to synthesize GTPαS A (Goody & Leberman, 1979). Using sensitive HPLC methods, we found this enzyme produced a GTPαS mixture of much the same A:B ratio as was obtained with phosphoglycerate kinase. As nucleosidediphosphate kinase suffers from the disadvantage that the product must be separated from a mixture of nucleotides, we have preferred the use of phosphoglycerate kinase. However, under limiting conditions of low ATP levels, and partially purified GDPαS A, nucleosidediphosphate kinase can be used to prepare GTPαS A in 100% isomeric purity.

GTPαS B can be produced at 85% diastereomeric enrichment with succinyl-CoA synthetase providing that, once again, one of the substrates (in this case succinyl-CoA) is limited. No triphosphate product was formed unless the product CoA was removed with phosphotransacetylase and acetyl phosphate. The succinyl-CoA synthetase reaction is reversible (Nishimura & Grinnell, 1972), and presumably this together with the nonenzymic hydrolysis of the unstable succinyl-CoA (Simon, 1957) prevents product accumulation unless the above precaution is taken. The ATP-dependent *E. coli* succinyl-CoA synthetase can produce ATP from ADP and acetyl phosphate (Hildebrand & Spector, 1969) and so can be used synthetically without succinyl-CoA. Unfortunately, no products were ob-

Table I: ^{31}P NMR Spectra of GTP and the Guanosine Phosphorothioate Analogues[a]

	α phosphorus	β phosphorus	γ phosphorus
GTP	−10.94 (d)	−21.48 (dd)	−5.92 (d)
GTPαS A	+42.55 (d)	−22.97 (dd)	−6.29 (d)
GTPαS B	+42.23 (d)	−22.08 (dd)	−5.41 (d)
GTPβS A	−12.03 (d)	+28.27 (dd)	−6.78 (d)
GTPβS B	−12.02 (d)	+28.27 (dd)	−6.85 (d)

[a] d = doublet and dd = doublet of doublets.

served when the GTP-dependent pig heart enzyme was incubated with a mixture of GDPαS A and B and acetyl phosphate. The GTPαS B formed above can be enriched to 95% purity by removal of the A diastereomer with phosphoglycerate kinase. The unfavorable equilibrium of this reaction must be displaced by converting the 1,3-diphosphoglycerate to 3-phosphoglyceraldehyde with phosphoglyceraldehyde dehydrogenase and condensing this aldehyde with semicarbazide. The enzymes used to prepare ATPαS B, creatine kinase and hexokinase, have extremely low activities with guanosine nucleotides (Crane, 1962; Watts, 1973) and would not be expected to be useful in the above synthesis. Several other enzymes including acetate kinase, pyruvate kinase, phosphofructokinase, glycerol kinase, gluconate kinase, and myosin showed activity with the α-phosphorothioates of GDP and GTP. However, these enzymes were not stereoselective enough to be synthetically useful.

The enzymatic syntheses of GTPβS A and B from GDPβS are straightforward. Pyruvate kinase produced GTPβS A and B in a 7:3 ratio, and purification was easily achieved by degradation of the B diastereomer with glycerol kinase. This method is similar to that used to produce ATPβS A except that much more pyruvate kinase was required and the back-digestion was performed with glycerol kinase rather than hexokinase. GTPβS B was prepared in exactly the same manner as ATPβS B by using acetate kinase and subsequently removing traces of GTP with myosin. Under our conditions, glycerol kinase, acetate kinase, and myosin show 100% stereoselectivities.

The synthesis of γ-^{32}P-labeled guanosine phosphorothioates also proved to be relatively easy. GTPαS A and B can be labeled exactly as for the corresponding adenosine analogues (Romaniuk & Eckstein, 1981) in what is essentially a modification of the Glynn & Chappell (1964) method for [γ-^{32}P]ATP production. γ-^{32}P-Labeled GTPβS can be produced from prochiral GDPβS and $^{32}P_i$ with succinyl-CoA synthetase. Once again the CoA formed has to be removed with acetyl phosphate and phosphotransacetylase in order for product to accumulate. The succinyl-CoA synthetase reaction was found to produce a 60:40 mixture of GTPβS B and A from prochiral GDPβS when the product was assayed for stereochemical purity with glycerol kinase and myosin (see Materials and Methods and below). These ratios are in reasonable agreement with the rates observed with GTPβS A and B in the reverse reaction catalyzed by this enzyme (Table II). Stereochemical purities of 95% could be obtained with removal of contaminating GTPβS A or B with myosin or glycerol kinase, respectively. The methods used to prepare [γ-^{32}P]ATPβS A and B (Romaniuk & Eckstein, 1981) can also be used for the GTPβS nucleotides.

The ^{31}P NMR chemical shifts of the phosphorothioates and GTP are given in Table I. Exchange of an oxygen by a sulfur results in a large downfield shift for the particular phosphorus group (Eckstein & Goody, 1976; Jaffe & Cohn, 1978b), and so this method confirms the location of the phosphorothioate group.

The purity of the GTPαS and GTPβS diastereomers was also checked by anion-exchange and reverse-phase HPLC. These two systems resolve GTPαS A and B and so give direct information about both chemical and stereochemical purity. After the enzymatic syntheses described, both GTPαS A and B were 95% pure, and the purity could be increased to 100% by the inclusion of a final chromatography step. When tested in these systems, the purity of [γ-^{32}P]GTPαS A or B was found to be essentially that of the unlabeled nucleotide starting material. Additionally, 98.5% of the counts applied coeluted with the product with the other 1.5% being $^{32}P_i$.

Both GTPβS A and B appeared to be 100% pure when tested in the above two HPLC systems. However, this only indicates 100% chemical purity and gives no information about stereochemical purity as these systems do not resolve the two diastereomers. In practice, a 50:50 mixture of GTPβS A and B always eluted as a single symmetrical peak in both HPLC systems with a wide variety of buffers of different compositions, pH values, and ionic strengths. Diastereomeric purity could be most easily determined enzymatically. GTPβS A and B were treated with myosin or glycerol kinase and the products of the reaction analyzed by anion-exchange HPLC (which resolved GTPβS A or B from any product GDPβS). The A diastereomer was completely converted to GDPβS with myosin whereas no reaction was observed with glycerol kinase. Conversely, GTPβS B is totally unreactive toward myosin but was completely converted to diphosphate with glycerol kinase. Taken together, these results, in which either no or 100% reaction occurs, give unequivocal proof that both GTPβS A and B are diastereomerically pure. Similar methods showed that [γ-^{32}P]GTPβS A or B was 98% chemically pure (with 2% of the counts eluting as $^{32}P_i$) and 95% diastereomerically pure.

The interaction of the purified GTPαS and GTPβS diastereomers with acetate kinase, RNA polymerase, and succinyl-CoA synthetase has been investigated, and the kinetic constants obtained are shown in Table II. Acetate kinase (Romaniuk & Eckstein, 1981) and RNA polymerase (Armstrong et al., 1979; Yee et al., 1979) have previously been investigated with the adenosine phosphorothioates, and very similar results have been found with both the adenosine and guanosine analogues. Thus, with acetate kinase, NTPβS B

Table II: K_m and V_{max} Values Obtained for Acetate Kinase, RNA Polymerase, and Succinyl-CoA Synthetase with GTP and the GTP Phosphorothioate Analogues

	acetate kinase		RNA polymerase		succinyl-CoA synthetase	
	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})
GTP	91.5	89.5	115	0.16	10	4.2
GTPαS A	95	40	135	0.047	150	0.045
GTPαS B	41	12.4			13	0.61
GTPβS A			220	0.078	45	2.5
GTPβS B	51	39.5	475	0.050	40	5.7

but not NTPβS A was a substrate, and NTPαS A was a better substrate than NTPαS B. Similarly, with RNA polymerase, NTPαS A but not NTPαS B was incorporated into polymer, and NTPβS A was slightly preferred as a substrate compared to NTPβS B. These results suggest that base substitution has a very minimal effect on the α- and β-phosphate binding domains of both enzymes. Furthermore, they allow the assignment of the absolute configurations of the guanosine analogues as the absolute configurations of the adenosine analogues are known (see references in the introduction). The activity of GTPβS B with acetate kinase means that this analogue has the same configuration around the β phosphorus as does ATPβS B and so is of the S_P configuration. The lack of activities seen with both GTPβS A and ATPβS A with this enzyme assigns the R_P configuration to this guanosine analogue. Similar arguments with the α-phosphorothioates and RNA polymerase require GTPαS A and GTPαS B to have the S_P and R_P configurations, respectively.

Succinyl-CoA synthetase has not been previously studied by the phosphorothioate method, and Table II shows that while all four analogues were substrates, the K_m and V_{max} values obtained differ from each other and from those for the natural substrate GTP. Both GTP and GTPαS B have similar K_m values whereas GTPαS A binds some 15 times more weakly. This suggests that the *pro-S* α-phosphate oxygen makes an important binding interaction, presumably a hydrogen bond, with the enzyme, whereas the *pro-R* oxygen is unimportant for good binding. The 4-fold increase in K_m values seen with GTPβS A and B indicates that both oxygen atoms on the β phosphorus also interact with the enzyme. Mechanistic studies with the *E. coli* ATP-dependent enzyme have shown that succinyl-CoA synthesis involves an initial transfer of the nucleotide γ-phosphoryl group to a histidine residue on the protein. This phosphohistidine residue then phosphorylates succinate to yield enzyme-bound succinyl phosphate, which in turn reacts with CoA to give succinyl-CoA (Nishimura & Meister, 1965; Hildebrand & Spector, 1969). If this mechanism holds for the GTP-dependent enzyme, then the α- and β-phosphorothioates only differ from each other and the natural substrate GTP in the first partial reaction, i.e., phosphohistidine formation. After this step all the nucleotides will obviously follow an identical reaction pathway. The similar V_{max} values seen with GTP and GTPβS A and B suggest that this first phosphoryl transfer is not rate limiting with these analogues, and so with the true substrate GTP either succinyl phosphate or succinyl-CoA formation must be the slowest step. The V_{max} is lowered about 10 times with GTPαS B and a further 10-fold with GTPαS A, and so with these analogues phosphohistidine formation obviously becomes rate limiting. A further reason for the very low rates seen with GTPαS A could be that the activating metal ion (in this case Mg^{2+}) binds to the α-phosphorus group. As Mg^{2+} normally binds to oxygen in the phosphorothioates, Mg·GTPαS A could form the incorrect metal nucleotide screw-sense isomer (Cornelius & Cleland, 1978) and so be a poor substrate. However, further studies with Co^{2+} (which binds to oxygen and sulfur) and Cd^{2+} (which binds to sulfur) must be carried out to confirm this assumption (Jaffe & Cohn, 1978a, 1979).

Conclusions

This paper presents simple methods for the preparation of GTPαS A and B as well as GTPβS A and B. Of the other guanosine phosphorothioates, the preparation of GDPαS A and B has been described (Goody & Leberman, 1979), and GDPβS and GTPγS are already commercially available. The latter two have been extensively used to study hormonal regulation of adenylate cyclase (Eckstein et al., 1979; Cassel et al., 1979a; Cassel et al., 1979b); GTPγS has been employed to excite photoreceptors (Fein & Carson, 1981) and to study the assembly of tubulin into microtubules (Kirsch & Yarbrough, 1981). GDPαS A and B have been applied for the study of elongation factor Tu–nucleotide interactions (Goody & Leberman, 1979). It is expected that the phosphorothioates described in this paper will also find use in these areas.

Acknowledgments

We thank G. Haag and U. Kutzke for valuable technical assistance and B. Seeger for recording the NMR spectra.

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Binding of a Glucagon Photoaffinity Label to Rat Liver Plasma Membranes and Its Effect on Adenylate Cyclase Activity before and after Photolysis†

Catherine Demoliou-Mason and Richard M. Epand*

ABSTRACT: The concentration-dependent stimulation of adenylate cyclase by the photoaffinity reagent 2-[(2-nitro-4-azidophenyl)sulfenyl]-Trp^{25}-glucagon (glucagon-NAPS) and also its binding characteristics were compared with those of the native hormone. The derivative was found to be slightly more potent in stimulating adenylate cyclase than glucagon, in the presence of guanosine 5′-triphosphate (GTP). ^{125}I-Labeled glucagon-NAPS or ^{125}I-labeled glucagon bound rapidly to receptors and was competitively displaced by unlabeled glucagon or glucagon-NAPS. Glucagon-NAPS displaced bound radiolabeled hormone at a lower concentration than did glucagon in the absence of GTP. Scatchard analysis of the binding data obtained from displacement of bound radiolabeled ligand with unlabeled peptide demonstrated a heterogeneous population of saturable glucagon binding sites. Glucagon-NAPS displayed a higher affinity (0.7 nM) for the high-capacity sites (80–90% of total binding sites) than glucagon (4.0 nM) in the absence of GTP. In the presence of the nucleotide, both ligands had approximately the same affinity (0.5–0.6 nM). Hill plot analysis of the binding data suggested noncooperative interactions. Photoaffinity labeling of plasma membranes with glucagon-NAPS resulted in an irreversible activation of adenylate cyclase with a reduced response to further stimulation by glucagon, glucagon-NAPS, and NaF.

One of the important mechanisms by which peptide hormones can initiate cellular response is through the binding of the hormone to specific, saturable receptor sites on the surface of plasma membranes of target cells (Insel, 1978; Levitzki & Helmreich, 1979; Rodbell, 1980). In the case of glucagon, it has been shown that the hormone binds reversibly to specific sites in liver plasma membranes at concentrations comparable to those required for stimulation of adenylate cyclase (Rodbell et al., 1971a, 1974; Lad et al., 1977; Welton et al., 1977). Guanosine 5′-triphosphate (GTP)[1] also affects adenylate cyclase activity and is required for activation by glucagon (Rodbell et al., 1971c, 1974). GTP lowers the affinity of the receptor for the hormone (Birnbaumer & Pohl, 1973; Lin et al., 1977).

Earlier studies from our laboratory have indicated that the hydrophobic amino acids of glucagon can be modified without loss of activity provided that the product is not polar (Epand, 1980; Epand et al., 1981). Nitrophenylsulfenyl derivatives of glucagon modified at the tryptophan-25 residue stimulated adenylate cyclase with the same or even higher potency than native glucagon (Epand & Cote, 1976; Wright & Rodbell, 1980). This property led to the synthesis of the photoreactive glucagon-NAPS derivative having a nitrophenyl azide moiety bound to position 2 of the indole ring of tryptophan at amino acid residue 25 of glucagon (Demoliou & Epand, 1980). Our purpose was to use this derivative for the photoaffinity labeling of the glucagon receptor. The criteria and advantages in using photoaffinity labeling over conventional affinity labeling have been discussed by Knowles (1972) and Bayley & Knowles (1977). Chemical modification of ligands with photoaffinity reagents has provided radiolabeled photoprobes which when

† From the Department of Biochemistry, McMaster University Health Sciences Centre, Hamilton, Ontario L8N 3Z5, Canada. *Received July 29, 1981.* These studies were supported by grants from the National Science and Engineering Research Council of Canada (A 9848) and the National Institutes of Health (AM 21285).

[1] Abbreviations: NAPSCl, (2-nitro-4-azidophenyl)sulfenyl chloride; glucagon-NPS, 2-[(2-nitrophenyl)sulfenyl]-Trp^{25}-glucagon; NAP-glucagon, N^{α}-(4-azido-2-nitrophenyl)glucagon; glucagon-NAPS, 2-[(2-nitro-4-azidophenyl)sulfenyl]-Trp^{25}-glucagon; ATP, adenosine 5′-triphosphate; cAMP, adenosine cyclic 3′,5′-phosphate; GTP, guanosine 5′-triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; BSA, bovine serum albumin; KIU, kallikrein inactivator units; NaDodSO$_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/82/0421-1989$01.25/0