THE PREPARATION OF THIOPHOSPHATE ANALOGS OF GDP AND THEIR INTERACTION WITH EF-Tu

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Received 27 March 1979

1. Introduction

The synthesis of pure diastereomers of ADP and ATP thiophosphate analogs has been described [1]. It has been demonstrated that enzymes which interact with these analogs do so, in almost all cases, with a more or less pronounced specificity for one diastereomer. Together with the determination of the absolute stereochemistry of the diastereomers of ATP(α-S), this has led to a description of the stereochemistry of nucleoside triphosphate polymerisation by DNA-dependent RNA polymerase [2]. The absolute stereochemistry of diastereomers of ATP(β-S) has been inferred from their interaction with hexokinase. The specificity of this enzyme for the isomers is reversed by replacing Mg^{2+} by Cd^{2+} indicating that, at least in this case, the structures of the metal complexes are responsible for the observed specificity [3].

There have been no reports on the preparation and properties of pure diastereomers of the corresponding analogs of guanosine nucleotides. These are potentially useful for several biological systems of current interest, including polypeptide elongation factors and tubulin. We report here the synthesis of the diastereomers of GDP(α-S) and their interaction with EF-Tu.

Abbreviations: NDP(α-S), nucleoside 5'-*O*-(1-thiodiphosphate); NTP(α-S), nucleoside 5'-*O*-(1-thiotriphosphate); NDP(β-S), nucleoside 5'-*O*-(2-thiodiphosphate); EF-Tu, polypeptide elongation factor Tu

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Elsevier/North-Holland Biomedical Press

2. Materials and methods

2.1. *Materials*

GMP(S) was prepared by the methods described for the preparation of AMP(S) [4], the mixed diastereomer of GDP(α-S) as described for ADP(α-S) [1], and GDP(β-S) as described for ADP(β-S) [5]. Myosin subfragment-1 was prepared by the method in [6], actin by the method in [7], and EF-Tu · GDP from *E. coli* (MRE 600) by the method currently used in this laboratory (Leberman et al., in preparation). Acetate kinase, lactate dehydrogenase, nucleoside diphosphate kinase and pyruvate kinase were obtained from Boehringer.

2.2. *Methods*

Nucleotides were separated analytically by thin-layer chromatography (TLC) on polyethyleneimine-impregnated cellulose sheets (Macherey and Nagel) developed with 0.75 M phosphate buffer (pH 7.0).

The reactivity of mixed and separated diastereomers with pyruvate kinase was determined from the rate of change in A_{340} of a solution of the thiophosphate analog together with 60 mM KCl, 40 mM Tris–HCl (pH 8.0), 1 mM phosphoenolpyruvate, 0.2 mM NADH, 5 mM $MgCl_2$, 0.2 mM dithiothreitol, 50 μg/ml lactate dehydrogenase, and the appropriate concentration of pyruvate kinase.

The diastereomers of GDP(α-S) were separated using the stereospecific transfer of phosphate from ATP in the presence of nucleoside diphosphate kinase. A solution containing 1.8 mM GDP(α-S) (mixed isomers), 20 mM ATP, 25 mM $MgCl_2$, 100 mM

Tris–HCl (pH 8.0), 2 mM dithiothreitol, and 25 μg nucleoside diphosphate kinase in 20 ml total vol. was incubated at 25°C. At 10 min intervals samples were analysed by TLC on PEI-cellulose in 0.74 M KH_2PO_4 (pH 3.4). After 100 min no further reaction was observed and ~50% of the GDP(α-S) had been converted to GTP(α-S). The mixture was applied to a column (2.5 × 20 cm) of QAE–Sephadex A-25. The column was developed with a 2 ml linear gradient of 0.3–0.6 M triethylammonium bicarbonate (pH 7.5). GDP(α-S) (isomer B) eluted behind but not completely separated from the large excess of ATP. GTP(α-S) (isomer A) eluted last and was well separated from the other nucleotides. Fractions containing the two analogs were concentrated by rotary evaporation and buffer salts removed by repeated evaporation with methanol. The residues from the two analogs were each dissolved in 1 ml 50 mM Tris–HCl (pH 7.8) containing 5 mM $MgCl_2$ and treated with ~1mg actomyosin subfragment-1 at room temperature until TLC indicated that the ATP in the GDP(α-S) (isomer B) fraction had been converted to ADP, and that GTP(α-S) (isomer A) had been converted to GDP(α-S) (isomer A). In both cases this required ~5 h. The isomers were further purified on columns (1.5 × 7 cm) of QAE–Sephadex A-25 developed with 1 l linear gradients of 0.15–0.4 M triethylammonium bicarbonate (pH 7.5). The yield of each isomer was ~11 μmol (the original yield of GTP(α-S) (isomer A) was 16 μmol).

The relative affinities of the thiophosphate analogs for EF-Tu were measured by competition with [^{3}H]GDP as in [8].

3. Results and discussion

Attempts to prepare pure isomers of GDP(α-S) and GTP(α-S) in useful quantities failed with pyruvate kinase because of the low reactivity of GDP(α-S). This was unexpected, since GDP is almost as good as ADP as a substrate for this enzyme. However, GDP(α-S) was phosphorylated 20-times slower than ADP(α-S) at 0.2 mM. By contrast, phosphorylation of GDP(α-S) by acetate kinase was facile, occurring at a similar rate to ADP(α-S). This reaction was not used preparatively since the apparent specificity for one isomer was not very pronounced. Phosphoryl transfer from a large excess of ATP to GDP(α-S) in the presence of nucleoside diphosphate kinase proved to be the method of choice, as the conditions under which phosphorylation proceeded to ~50% were easily found. By analogy with ADP(α-S), isomer A was converted to GTP(α-S) which could be degraded by actomyosin-S1 to GDP(α-S) (isomer A). The remaining GDP(α-S) was assumed to be diastereomer B, also by analogy with ADP(α-S). The diastereomers showed the expected specificity with respect to pyruvate kinase, isomer A being phosphorylated ~15-times faster than isomer B at 16 μM substrate. Contamination of diastereomer B with diastereomer A was judged to be <10% using the pyruvate kinase reaction.

EF-Tu binds GDP very strongly and the exchange of the GDP normally bound to the isolated enzyme, for [^{3}H]GDP, is a common assay for the protein [9]. Figure 1 shows the titration curves obtained with varying concentrations of GDP and thiophosphate analogs in such an assay system. The control curve with GDP shows that at a concentration equal to that of the [^{3}H]GDP only ~50% of the radioactivity is bound to the protein. With GDP(α-S) (isomer B) and GDP(β-S) the concentration of the analog has to be ~50-times greater to obtain this condition. With GDP(α-S) (isomer A) a concentration only 1.5-times greater than that of [^{3}H]GDP is required for 50% inhibition. More exact estimates of the ratios of the binding constants were obtained from plots of [Tu · GDP analog] / [Tu · GDP] against [GDP analog] / [GDP]. The slopes of the resulting straight lines gave the ratios K_{analog}/K_{GDP}, where K is the respective association constant. The values obtained were 0.62, 0.023, 0.021, for GDP(α-S) isomer A, GDP(α-S) isomer B, and GDP(β-S), respectively. The values for GDP(β-S) and GDP(α-S) isomer B should be regarded as upper limits because of possible contamination by GDP and GDP(α-S) isomer A, respectively. It can be seen that the A-diastereomer of GDP(α-S) is bound considerably more strongly than the B-diastereomer, its affinity being only slightly lower than GDP. Since the binding of GDP to EF-Tu is stimulated by the formation of its Mg^{2+} complex [10], the structure of the required complex can be regarded as analogous to that of MgGDP(α-S) isomer A (see fig.2).

While the much weaker binding of the GDP(α-S) isomer B is understandable in terms of the spatial orientation of the groups around the α-phosphorus

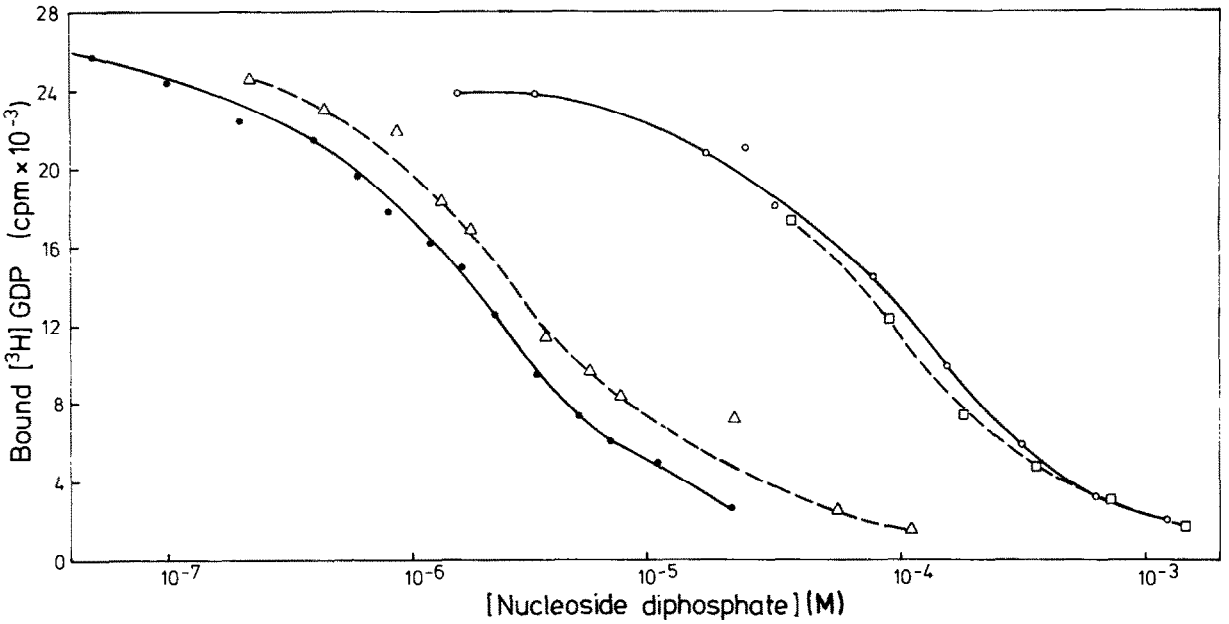

Fig.1. Inhibition of [^{3}H]GDP binding to EF-Tu. Samples (1 ml) of various concentrations of either GDP or analog were incubated at room temperature for 2 h with 120 nM EF-Tu · GDP and 2 μM [^{3}H]GDP in 50 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 0.5 mM dithiothreitol. After 2 h the samples were filtered through nitrocellulose filters (Schleicher and Schuell, BA85) and the amount of radioactivity retained by the filters measured. (–●–●–) GDP; (–△–△–) GDP(α-S) isomer A; (–□–□–) GDP(α-S) isomer B; (–○–○–) GDP(β-S).

in the Mg^{2+} complexes of the two diastereomers, the weak binding of GDP(β-S) is more surprising. Assuming that the configuration at the α-phosphorus in the Mg^{2+} complex must correspond to the A-diastereomer of GDP(α-S), two possible configurations of groups in the diphosphate remain due to the presence of a new chiral centre at the β-phosphorus on formation of the Mg^{2+} complex. These are shown in fig.3. Since neither of these structures is well recognised by EF-Tu, it appears that the interaction with the enzyme at the β-phosphate involves both non-complexed oxygens in MgGDP, this interaction being weakened in GDP(β-S) regardless of the relative orientations of oxygen and sulphur.

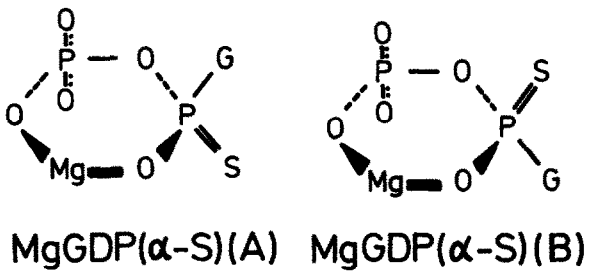

Fig.2. Structures of the magnesium complexes of the diastereomers of GDP(α-S) by analogy with ADP(α-S) [2].

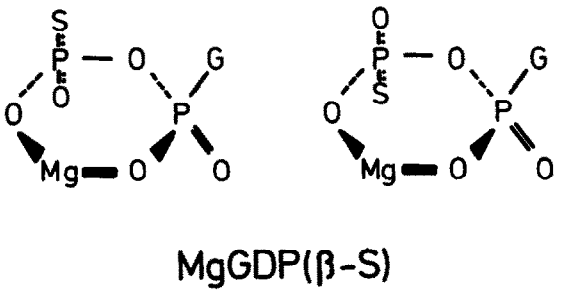

Fig.3. Structures of two diastereomers of the magnesium complex of GDP(β-S). A further pair with the opposite configuration at the α-phosphorus can also exist.

Acknowledgements

We would like to thank Maria Isakov and Renate Schumann for excellent technical assistance.

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