Characterization of the GTPase Reaction of Elongation Factor Tu

DETERMINATION OF THE STEREOCHEMICAL COURSE IN THE PRESENCE OF ANTIBIOTIC X5108*

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The stereochemical course of the GTPase of elongation factor Tu from Escherichia coli has been determined by making use of the reaction dependent on antibiotic X5108 (an N-methylated derivative of kirromycin). Guanosine 5'-ô-thio)triphosphate stereospecifically labeled with 17O and 18O in the ô-position was hydrolyzed in the presence of elongation factor Tu and X5108. The configuration of the product, inorganic 5'-ô-thiotriphosphate was analyzed by 31P NMR after its stereospecific incorporation into adenosine 5'-ô-thiotriphosphate. The analysis showed that the hydrolysis proceeds with inversion of configuration at the transferred phosphorus, implying that there is not a phosphoencezyme intermediate.

Overall retention is explained simply by two steps: the formation and breakdown of a phosphotransfer intermediate. This stereochemical technique has been applied previously to three ATPases, myosin, mitochondrial and sarcoplasmic reticulum, and the results support this explanation for triphosphatases (Webb and Trentham, 1978, 1981; Webb et al., 1977). Each phosphotransfer step probably occurs with inversion, so that overall inversion implies a single step via a direct, in-line mechanism (Knowles, 1980). Overall retention is explained most simply by two steps: the formation and breakdown of a phosphorylated intermediate. This stereochemical technique has been applied previously to three ATPases, myosin, mitochondrial and sarcoplasmic reticulum, and the results support this explanation for triphosphatases (Webb and Trentham, 1980).

Whether this reaction occurs with retention or inversion of configuration can be determined (Webb and Trentham, 1980a; Webb, 1982). There is strong evidence that the stereochemical courses of triphospho and phospho transfer are identical (Eckstein et al., 1982), so that the result with GTPyS should give information as to whether or not GTP hydrolysis occurs via a phosphorylated intermediate, such as a phosphoencezyme.

The partial reactions of EF-Tu (Wolf et al., 1977) have shown that this GTP hydrolysis shares common features with the physiological reaction, so that it is probable that the two reactions share a common active site and chemical mechanism. In this paper, we make use of this uncoupled hydrolysis to determine the stereochemical course of EF-Tu-catalyzed GTPase activity, in the presence of antibiotic X5108, an N-methylated derivative of Kirromycin. X5108 binds tightly to EF-Tu (Wilson and Cohn, 1977; Eccleston, 1981a) and has similar effects as kirromycin itself on the partial reactions of EF-Tu (Wolf et al., 1977).

Using oxygen exchange methods, we have investigated the possibility that the cleavage step of the EF-Tu GTPase mechanism is reversible. The results are compared with those obtained with EF-G, the elongation factor involved in the translocation process of the peptide elongation cycle.

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† The abbreviations used are: EF-Tu, elongation factor Tu; EF-G, elongation factor G; GTPyS, guanosine 5'-ô-thiotriphosphate; ATPyS, adenosine 5'-ô-thiotriphosphate; ATPyS, adenosine 5'-ô-thiotriphosphate.

To avoid confusion in molecular structures, π-bonds, negative charges, and protonation states are omitted.
**EXPERIMENTAL PROCEDURES**

EF-Tu-GTP from Escherichia coli was prepared and characterized as described by Eccleston (1981). EF-G and ribosomes from E. coli were prepared as described by Webb and Eccleston (1981). Antibiotic X5108 was from Hoffmann-La Roche and used as a 25 mM solution in ethanol. (γ-S) [32P]GTP, [γ-'O]GTP, [3H]GTP, [32P]GTPyS, [3H]GDP, [32P]GDP, 200 μM GTPyS, 250 μM X5108, 0.2 μM units-1 of guanylate kinase, 5 units-μl-1 of nucleosidediphosphate kinase, and 200 μM ADP was incubated at 37°C. The column eluate was mixed, using a Valco zero volume-T connection, with a Waters system and a Whatman Partisil 10/25 column (25 cm × 1 cm × 0.025 cm). The rate of hydrolysis was determined using high performance liquid chromatography as described by Webb and Eccleston (1981). In addition, formation of inorganic thiophosphate was determined by high performance liquid chromatography using conditions described by Webb and Eccleston (1981). In order to determine that the only GTPase activity in our EF-Tu preparation was the reaction dependent on X5108, the rates of GTP hydrolysis were measured in the absence and presence of the antibiotic. In the absence of X5108, there was no observable hydrolysis (<33, μM·h-1). In its presence, the rate was approximately 1000 μM·h-1. This is equivalent to a rate constant of 0.03 s-1, so that hydrolysis is limited probably by the rate of GDP release from EF-Tu, consistent with the results of Pasano et al. (1978).

GTPyS hydrolyzes slowly in the presence of EF-Tu and antibiotic X5108, as shown in Table I. There is also a significant background breakdown, in the absence of the protein, consistent with the instability of GTPyS solutions. The rate of breakdown in the presence of EF-Tu but without X5108 was similar to the background rate. The products of these background reactions were not determined, although it is probable that, at least in part, the GTPyS is destroyed by loss of sulfur. With X5108, the rate of disappearance of GTPyS is 4-fold larger in the presence of EF-Tu than in its absence. The products in this case were shown by high performance liquid chromatography to be GDP and inorganic thiophosphate. However in the absence of the coupling system consisting of ADP, guanylate kinase, and nucleoside diphosphate kinase, which converts GDP to GMP, the rate of hydrolysis was consistently slower than in its presence and the hydrolysis further slowed down after partial reaction. This is probably due to inhibition by GDP (Miller and Weissbach, 1977). Hence the coupling system was added in all reactions in Table I, except where indicated, so that there was no significant GDP accumulation. GMP forms and binds at least 104 times more weakly to EF-Tu than does GDP (Miller and Weissbach, 1977).

The ability of EF-Tu to catalyze oxygen exchange in the absence of antibiotic or ribosomes was tested using [32P]GTP, [γ-'O]GTP, enriched to an extent of 97% in each labeled position. This GTP was synthesized and the 'O distribution about its β and γ phosphorus atoms was determined by 31P NMR as previously described (Webb, 1980). Nucleotide-free EF-Tu was prepared as described by Eccleston et al. (1981), except that EDTA was not removed. It contained 20% residual GDP. Each of the two phosphates was labeled with 31P NMR as previously described (Webb, 1980).

**RESULTS AND DISCUSSION**

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**TABLE I**

| Additions | Steady state rate (μM·h-1) |
|-----------|--------------------------|
| EF-Tu     | 14                       |
| X5108     | 21                       |
| X5108 + EF-Tu | 54                  |
| X5108 + EF-Tu, without coupling system | 45 |

The instability of GTPyS is apparent here because of the relatively slow rate of hydrolysis in the presence of EF-Tu and X5108 and the elevated temperature. In most other triphosphates studied with nucleoside γ-thiotriphosphates, the rate of catalyzed hydrolysis is much larger, so that the background breakdown is unimportant (Webb, 1982).
whether the hydrolysis proceeded with retention or inversion of configuration. Its configuration was determined after incorporation into ATP\(\beta\)S by \(^{31}\)P NMR of the \(\beta\)-phosphorus (Webb and Trentham, 1980a). The formation of ATP\(\beta\)S is highly stereoselective, but one oxygen is lost from the thiomophosphate during the incorporation. One third of the molecules lose \(^{17}\)O, one third lose \(^{18}\)O, and one third lose \(^{16}\)O. Molecules that retain \(^{17}\)O are not visible in the NMR spectrum due to quadrupole broadening of the \(\beta\)-phosphorus signal by the nuclear spin of \(^{17}\)O. Molecules that lose \(^{18}\)O are visible and give rise to ATP\(\beta\)S containing an \(^{16}\)O which is nonbridging (species 1) or bridging (2) depending on whether it is derived from \(S\) or \(R\) inorganic thiophosphate:

\[
\begin{align*}
\text{S} & \quad \longrightarrow \quad A - O - P - O - P - O - P - O \\
\text{R} & \quad \longrightarrow \quad A - O - P - O - P - P - O - P - O
\end{align*}
\]

These two ATP\(\beta\)S species are distinguished by \(^{31}\)P NMR, due to slightly different chemical shifts (Webb and Trentham, 1980a).

Isotopic enrichments are less than 100% in the GTP\(\gamma\)S so that there are other inorganic thiophosphate species, partially labeled and unlabeled, apart from the \(^{16}\)O, \(^{17}\)O, \(^{18}\)O-labeled, chiral species. These give rise to other peaks in the \(^{31}\)P NMR spectrum of ATP\(\beta\)S apart from those due to species 1 or 2. This spectrum is shown in Fig. 1, in which the peaks are assigned to the various ATP\(\beta\)S species as described by Webb and Trentham (1980a). Qualitatively the excess ATP\(\beta\)S with bridging \(^{16}\)O over that with nonbridging \(^{18}\)O shows that the major pathway for GTP\(\gamma\)S hydrolysis is inversion, Table II shows the relative peak intensities from this spectrum, compared to those calculated for inversion or retention. Once the lack of isotopic purity of ATP\(\beta\)S is taken into account, the spectrum is close to that calculated for all GTP\(\gamma\)S hydrolysis occurring with inversion.

It is probable that the hydrolysis of GTP, catalyzed by EF-Tu, has the same chemical mechanism as GTP\(\gamma\)S and so occurs with inversion of configuration at the transferred phosphorus atom. This is strong evidence against a phosphorylated intermediate and is consistent with the reaction occurring in a single step, with direct, in-line displacement of GDP by a water oxygen.

This mechanism of the GTPase was investigated further by oxygen isotope experiments to detect transient cleavage of GTP on EF-Tu. Although EF-Tu does not catalyze significant hydrolysis of GTP in the absence of ribosomes or kiromycin, the possibility remains that reversible hydrolysis occurs as in Equation 3 but that no product is released.

\[
\text{EF-Tu \cdot GTP} \rightleftharpoons \text{EF-Tu \cdot GDP} + \text{Pi}
\]

If this were the case, the EF-Tu system would parallel actomyosin, since myosin catalyzes the rapid, reversible cleavage of ATP even in the absence of actin (Taylor, 1979). To detect such transient, reversible cleavage we used GTP labeled with \(^{18}\)O as in species 3:

When this GTP was incubated with EF-Tu for 4 h at 0 °C, no change occurred to the pattern or extent of labeling, as determined by \(^{31}\)P NMR. This implies that no GTP is cleaved reversibly on the protein: that is, it is not cleaved transiently to EF-Tu \(\cdot\) GDP \(\cdot\) P, then re-formed to EF-Tu \(\cdot\) GTP. If this transient reaction with water occurred, the labeling would have changed probably in two ways, shown in species 4 of Equation 4. In one process, oxygen exchange with the water would have occurred. This exchange would occur on transient cleavage, since the first-formed P, is \(\text{PO}_{4}\). On re-forming GTP, there is a chance that \(^{18}\)O will be lost from the molecule resulting in only partial \(^{18}\)O enrichment (O) remaining in the \(\gamma\)-phosphate. Also, the transient cleavage would probably give rise to positional isotope exchange (Midelfort and Rose, 1976). The GTP would be cleaved between the \(\beta\)-\(\gamma\)-\(^{18}\)O and the \(\gamma\)-P. The \(^{18}\)O remaining on GDP would become equivalent with the other \(\beta\)-oxygen (\(^{16}\)O). On re-forming GTP, one of these \(\beta\)-

![Diagram](http://www.jbc.org/)

**FIG. 1.** \(^{31}\)P NMR spectrum of the \(\beta\)-phosphorus of ATP\(\beta\)S. The ATP\(\beta\)S was derived from inorganic thiophosphate, product of \((\gamma\)-S \([\beta\gamma'\text{O}\gamma'\text{O}]\text{GTP}\gamma\)S hydrolysis in the presence of X5108 and EF-Tu.

**TABLE II**

| Relative peak intensities |
|--------------------------|
| Unlabeled | \(^{17}\)O, bridging | \(^{18}\)O, non-bridging | \(^{16}\)O |
| Calculated if retention | 28 | 25 | 37 | 10 |
| Calculated if inversion | 28 | 37 | 25 | 19 |
| Observed | 26 | 24 | 26 | 12 |

*These spectra are calculated from the known isotopic enrichments of the GTP\(\gamma\)S; 85% for \(^{16}\)O, 45% for \(^{18}\)O. The \(^{17}\)O positions also contain 30% \(^{18}\)O. The calculation assumes a 5% loss of isotope during the hydrolysis of GTP\(\gamma\)S or during incorporation of inorganic phosphosphate into ATP\(\beta\)S, as described by Webb et al. (1980), to obtain the observed \(^{18}\)O-enrichment in ATP\(\beta\)S. No attempt was made to determine the extent of exchange during hydrolysis of GTP\(\beta\)S since all such determinations in other systems (Webb, 1982) have found negligible extents of exchange. The noise in the spectrum causes an uncertainty of approximately ±1% in the observed peak intensities.\n
*The peak intensities have small corrections due to peak overlap.
Stereochemistry of Elongation Factor Tu GTPase

Oxygens (180, 18O) of GDP becomes the β-γ-bridging oxygen of GTP. This bridging oxygen, therefore, would be 18O in some molecules.

Both oxygen exchange with water and positional isotope exchange can be detected by the change in 18O labeling measured by 31P NMR. Since no changes from 3 to 4 occurred, it is probable that no reversible, transient cleavage occurred.4 The cleavage is only facilitated on interaction with ribosomes or kirromycin, when the direct displacement of GDP from GTP by water occurs. This behavior is comparable to that of EF-G, which also catalyzes GTP hydrolysis (in the presence of ribosomes) with inversion of configuration (Webb and Eccleston, 1981). It also does not seem to catalyze even transient cleavage of GTP in the absence of ribosomes since a similar experiment with 18O-labeled GTP and EF-G, as described under "Experimental Procedures," showed no change in the pattern or extent of labeling.

It has been shown also that there is no intermediate oxygen exchange with water during GTP hydrolysis in the presence of EF-Tu (from Bacillus stearothermophilus) and X5108. This is most likely due to the cleavage being irreversible although product release can occur. A similar lack of exchange with water has been observed for GTP hydrolysis in the presence of EF-G and a saturating concentration of ribosomes (Rohrbach et al., 1974). In this case, the rate of hydrolysis increases with increasing ribosome concentrations. However, no exchange occurs over a wide range of activation as described under "Experimental Procedures." Thus, the cleavage is irreversible even at low activation, when the velocity of product release is low.

There is therefore, a close similarity in the mechanism of the uncoupled GTPase of the two elongation factors. Both catalyze the hydrolysis with inversion of configuration and the cleavage appears to be irreversible. It is probable that the cleavage cannot occur in the absence of activating kirromycin or ribosomes. This description contrasts with that of myosin, which catalyzes the rapid, reversible cleavage of ATP, with inversion of configuration, in the absence of actin. The overall hydrolysis rate is increased by the presence of actin, which increases the rate of product release, but has only a small effect on the rate of cleavage (Stein et al., 1981). Cleavage becomes effectively irreversible only at high actin concentrations.

It is not yet understood how these differences in reversibility of the cleavage step of the two energy-transducing systems (the cross-bridge of muscle and the elongation cycle of protein biosynthesis) are related to the biological function. However, nucleotide binding and cleavage clearly have large effects on the protein-protein interactions in both systems.

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4 This is the most likely explanation of the result. It is possible that cleavage occurs but that all β and γ oxygens are held rigid (for example, by tight metal coordination) so that the phosphates cannot rotate. If such rotation cannot occur, the oxygens cannot exchange positions.

5 J. S. Taylor and M. Cohn, personal communication.
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