Regulatory Mechanisms Differ in UMP Kinases from Gram-negative and Gram-positive Bacteria*

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In this work, we examined the regulation by GTP and UTP of the UMP kinases from eight bacterial species. The enzyme from Gram-positive organisms exhibited cooperative kinetics with ATP as substrate. GTP decreased this cooperativity and increased the affinity for ATP. UTP had the opposite effect, as it decreased the enzyme affinity for ATP. The nucleotide analogs 5-bromo-UTP and 5-iodo-UTP were 5–10 times stronger inhibitors than the parent compound. On the other hand, UMP kinases from the Gram-negative organisms did not show cooperativity in substrate binding and catalysis. Activation by GTP resulted mainly from the reversal of inhibition caused by excess UMP, and inhibition by UTP was accompanied by a strong result mainly from the reversal of inhibition caused by excess 5-bromo-UTP and 5-iodo-UTP were 5–10 times stronger inhibitors than the parent compound. On the other hand, UMP kinases from the Gram-negative organisms did not show cooperativity in substrate binding and catalysis. Activation by GTP resulted mainly from the reversal of inhibition caused by excess UMP, and inhibition by UTP was accompanied by a strong increase in the apparent K_m for UMP. Altogether, these results indicate that, depending on the bacteria considered, GTP and UTP interact with different enzyme recognition sites. In Gram-positive bacteria, GTP and UTP bind to a single site or largely overlapping sites, shifting the T ⇌ R equilibrium to either the R or T form, a scenario corresponding to almost all regulatory proteins, commonly called K systems. In Gram-negative organisms, the GTP-binding site corresponds to the unique allosteric site of the Gram-positive bacteria. In contrast, UTP interacts cooperatively with a site that overlaps the catalytic center, i.e. the UMP-binding site and part of the ATP-binding site. These characteristics make UTP an original regulator of UMP kinases from Gram-negative organisms, beyond the common scheme of allosteric control.

Bacterial UMP kinases represent a particular subfamily of NMP kinases (1, 2). They do not share any significant sequence homology with other known NMP kinases and exist in solution as stable hexamers. A first structural model of Escherichia coli UMP kinase (3) based on the conservation of the carbamate kinase and N-acetylglutamate kinase folds (4, 5) helped to better rationalize previous site-directed mutagenesis experiments (6). The crystal structure of E. coli UMP kinase (7) indicated a similar fold between its monomers and N-acetylglutamate kinase, a dimeric enzyme (4, 5). However, the quaternary structure assembly of these two proteins is completely different (7). Deposited crystal structures of UMP kinases from other bacteria such as Pyrococcus furiosus (8), Neisseria meningitidis (Protein Data Bank code 1YBD), Hemophilus influenzae (code 2A1F), and Streptococcus pyogenes (code 1Z9D) show three-dimensional structures very similar to that of the E. coli enzyme. The residues essential for binding nucleotide substrates and catalysis are conserved among all bacterial UMP kinases (Fig. 1) (3, 9). Consequently, the active sites of these enzymes and the phosphoryl transfer mechanisms are most probably similar.

Comparison of the biochemical properties of recombinant UMP kinases from Gram-negative E. coli (1, 2) and Gram-positive Streptococcus pneumoniae (10) indicated significant differences in their kinetic properties particularly in their regulation by nucleotides. Unlike the E. coli enzyme, UMP kinase from S. pneumoniae exhibited cooperative kinetics with respect to ATP, and its activation by GTP resulted in a decrease in cooperativity and an increase in affinity for ATP.

To substantiate and eventually extend these observations to other UMP kinases from Gram-negative or Gram-positive bacteria, the corresponding pyrH genes were cloned, and the recombinant proteins were studied for their kinetic properties in both forward and reverse reactions. Thus, GTP and UTP are so far effectors for all the investigated UMP kinases. They act on the kinetic parameters mostly via conformational changes induced in the protein. Consequently, the regulating effects of GTP and UTP on UMP kinases from both Gram-negative and Gram-positive organisms are strongly related to the quaternary structures of these proteins.

EXPERIMENTAL PROCEDURES

Chemicals—Nucleotides, restriction enzymes, T4 DNA ligase, Vent and Tfi DNA polymerases, and coupling enzymes were purchased from Roche Applied Science, New England Biolabs, Qiogene Inc., or Sigma. UTP and UMP analogs halogenated at position 5 in the heterocycle were purchased from Jena Bioscience GmbH. NDP kinase from Dictyostelium...
discoideum (2000 units/mg of protein) was kindly provided by I. Lascu.

Bacterial Strains, Plasmids, Growth Conditions, and DNA Manipulations—General DNA manipulations were performed as described by Sambrook et al. (11). Open reading frames from the pyrH gene from different organisms (E. coli, Salmonella typhimurium, H. influenzae, N. meningitidis, B. subtilis, S. pneumoniae, S. aureus, and E. faecalis) were amplified from chromosomal DNA as template using the corresponding primers (Table 1). The PCR products were inserted into the vector pET24a (between the NdeI and EcoRI restriction sites) or the vector pET28a (between the NdeI and XhoI or HindIII restriction sites) (Novagen). The resulting plasmids were introduced into strain BL21(DE3)/pDIA17 (12) to overproduce the UMP kinase. The recombinant strains were grown in 2YH medium supplemented with kanamycin (70 μg/ml) and chloramphenicol (30 μg/ml) to an absorbance of 1.5 at 600 nm, and then overproduction was triggered by isopropyl β-D-thiogalactopyranoside induction (1 mM final concentration) for 3 h at 37°C. The cells were harvested by centrifugation and served as a source for protein purification.

The single mutants T135A and N137A and the double mutant T135A/N137A of B. subtilis UMP kinase were constructed by the one-tube PCR-based mutagenesis method (13) using the plasmid harboring the corresponding UMP kinase gene as template, Tfu DNA polymerase, the dNTPs, and the following mutagenic oligonucleotides: T135A, 5'-GGAATATGGGTTTCCAGCGCCCGCAG-3'; and N137A, 5'-AGTTGAGAAATATGGAGCTCCTGTGCCCGCAG-3'.

**FIGURE 1.** Shown is the sequence alignment of eight bacterial UMP kinases (UMPK) explored in this work and belonging to Gram-negative (E. coli (Ec), S. typhimurium (St), H. influenzae (Hi), and N. meningitidis (Nm)) and Gram-positive (B. subtilis (Bs), S. pneumoniae (Sp), S. aureus (Sa), and E. faecalis (Ef)) organisms. Conserved residues are highlighted in gray. The residues deduced in E. coli UMP kinase as interacting with different nucleotides are shown in yellow (ATP), blue (UMP), and magenta (GTP). Asterisks indicate residues modified by site-directed mutagenesis either in the past or in the present work.
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ATGGAGCTCCAGCGCCGACGCGAAAAT-3’. The PCR product was cloned at the NdeI and Xhol restriction sites of the pET28a vector. All plasmids were sequenced to verify either their integrity or the incorporation of the desired modifications.

Purification of UMP Kinases and Activity Assay—The different N-terminally His-tagged UMP kinases (E. coli elliN soluble variant, H. influenzae, E. coli, B. subtilis, S. pneumoniae, S. aureus, and E. faecalis) overproduced in E. coli were purified by nickel-nitrilotriacetic acid affinity chromatography using the QIAexpress expression system (14). The recombinant proteins (purity >95% as indicated by SDS-PAGE) were stored at +4 °C in buffer (pH 8.0) containing 50 mM Na₂HPO₄, 150 mM imidazole, and 300 mM NaCl. Recombinant S. typhimurium UMP kinase was purified as described previously for wild-type UMP kinase from E. coli (1). Protein concentration was measured according to Bradford (15). Ion spray mass spectra of purified proteins were recorded on an API-365 quadrupole mass spectrometer (PerkinElmer Life Sciences) equipped with an ion spray (nebulizer-assisted electrospray) source. SDS-PAGE was performed as described by Laemmli (16).

UMP kinase activity was determined at 30 °C using coupled spectrophotometric assays (0.5-ml final volume) on an Eppendorf ECOM photometer (17). The reaction medium in the forward direction contained 50 mM Tris-HCl (pH 7.4); 50 mM KCl; 1 mM phosphoenolpyruvate; 0.2 mM NADH; 2 units each of hexokinase and glucose-6-phosphate dehydrogenase; and various concentrations of MgCl₂, ATP, and UMP. The UMP kinase appropriately diluted in 50 mM Tris-HCl (pH 7.4) was then added, and the increase in absorbance was recorded at 340 nm. The reaction medium in the reverse direction contained 50 mM Tris-HCl (pH 7.4); 50 mM KCl; 1 mM glucose; 0.4 mM NADP⁺; 2 units each of hexokinase and glucose-6-phosphate dehydrogenase; and various concentrations of MgCl₂, ADP, and UDP. The appropriately diluted UMP kinase was then added, and the increase in absorbance was recorded at 340 nm. One unit of UMP kinase corresponds to 1 μmol of product formed per min.

The thermal stability of UMP kinases was tested by incubating the purified enzymes (1 mg/ml) in 50 mM Tris-HCl (pH 7.4 or 8.5) containing 0.1 M NaCl at a temperature between 30 °C and 80 °C for 10 min in the presence or absence of various nucleotides. The results (expressed as the percentage of residual activity compared with non-incubated controls) were used to calculate the temperature of half-inactivation (Tₜₐ₃₂).

Calculation of Magnesium-Nucleotide Complexes and Kinetic Data Analysis—The concentration of MgCl₂ in the assay medium in which coexisted nucleotides differing in the number of phosphate units was found to be critical for several reasons. The dissociation constant (Kₐ) of metal-nucleotide complexes varies within 2 orders of magnitude from 0.1 mM for MgNTP, 1 mM for MgNDP, and 20 mM for MgNMP (18, 19). On the other hand, as some nucleotides played multiple roles, this resulted in mixed kinetic effects. Numerical simulations with different concentrations of MgCl₂ and nucleotides showed that an acceptable compromise in the forward reaction was to use a 2 mM excess of MgCl₂ above the concentration of NTPs. Thus, for the range of ATP (0.2–25 mM) and UMP (0.1–2 mM) concentrations used in most experiments, MgATP represented 95.7 ± 0.9% of the total ATP, and magnesium-free UMP represented 89.6 ± 2% of the total UMP (Table 2). Furthermore, the free metal ion (between 1.8 and 2.8 mM) was held at a sufficiently high but non-inhibitory concentration. For the sake of simplicity, the calculation of the kinetic constants in the forward reaction employed the actual concentration of various nucleotides. In this case, a Kₐ or Kₐₕₙₜ for MgATP corresponds approximately to a Kₐₚ for MgATP of 1.9 mM. Similarly, a Kₐ for UMP of 0.1 mM corresponds approximately to a Kₐₚ of 0.09 mM for magnesium-free UMP. In the reverse reaction, the concentration of MgCl₂ (millimolar) was related to the concentrations of UDP and ADP (or GDP when present) by the following relationship: [MgCl₂]₀ = 4 + 0.8 [NDP]. Under these conditions, the concentration of MgNDPs represented 80% of the total nucleotide concentration, and the concentration of free magnesium cation was always 4 mM. When GMP or GMP-PNP was used, this relation changed as follows: [MgCl₂]₀ = 4 + 0.8 [NDP] + 0.1 [NMP], and [MgCl₂]₀ = 4 + 0.8 [NDP] + [NTP], respectively. With these empirical adjustments, the [MgNDP]/[NDP] ratio varied by <1%, whereas the concentration of MgNTP represented 98% of the total NTP.

### TABLE 1

| Organism            | Flanking primers | Restriction sites | Ref. |
|---------------------|------------------|------------------|------|
| E. coli             | 5’-GGGAATTCCTAAATACGCTCAATGCAAAACCCGTAT-3’ | NdeI and XhoI | 1    |
| S. typhimurium      | 5’-GGGATCCGATCTCAGTCAGTCAGTCATTATTAAGTCCCTTTCAT-3’ | NdeI and HindIII | This study |
| H. influenzae       | 5’-CCCCCAGGATCCAGCAGCAGACAGCCCTTAC-3’ | NdeI and EcorI | This study |
| N. meningitidis     | 5’-GGGATCCGATCTCAGTCAGTCAGTCATTATTAAGTCCCTTTCAT-3’ | NdeI and XhoI | This study |
| S. pneumoniae       | 5’-GGGATCCGATCTCAGTCAGTCAGTCATTATTAAGTCCCTTTCAT-3’ | NdeI and XhoI | This study |
| S. aureus           | 5’-GGGATCCGATCTCAGTCAGTCAGTCATTATTAAGTCCCTTTCAT-3’ | NdeI and HindIII | This study |
| E. faecalis         | 5’-GGGATCCGATCTCAGTCAGTCAGTCATTATTAAGTCCCTTTCAT-3’ | NdeI and HindIII | This study |
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The kinetic results were fitted to one of the following three equations by nonlinear least-squares fitting analysis using KaleidaGraph software (Equations 1–3),

\[ v = \frac{V_m[S]}{(K_m + [S])} \]  
\[ v = \frac{V_m[S]}{(K_m + [S] + [S]^2/K)} \]  
\[ v = \frac{V_m[S]^n}{(K_{0.5} + [S]^n)} \]

where \( v \) is the steady-state velocity, \( V_m \) is the maximal rate, \([S]\) is the substrate concentration (i.e. ATP or UMP in the forward reaction and ADP or UDP in the reverse reaction), \( K_m \) is the Michaelis-Menten constant, \( K_{0.5} \) is the substrate concentration at half-saturation, \( K_i \) is the inhibition constant, and \( n \) (or \( n_i \)) is the Hill number (indicating the cooperativity index). The accuracy of the constants calculated by these fittings (on average, they varied within ±10%) depended on the experimental errors (protein concentration and stability, purity of the commercially available nucleotides, and efficiency of the coupling enzymes in the assay system) and the computed concentration of the “active” metal-free or metal-complexed nucleotides from the corresponding dissociation constants.

RESULTS

Purification and Specific Activity of Recombinant UMP Kinases—Because we did not observe significant differences in the specific activities of the wild-type or His-tagged forms of E. coli (7), H. influenzae (this work), and B. subtilis (9) UMP kinases, the recombinant enzyme from the other bacterial species was overproduced with an N-terminal His tag and purified by affinity chromatography on nickel-nitrilotriacetic columns. We assumed that the His tag does not affect the activity of other bacterial UMP kinases. Gel permeation chromatography on Sephacryl S-300 and ultracentrifugation by sedimentation equilibrium confirmed that all variants exist as hexamers.

Table 3 indicates the specific activity of UMP kinases from eight bacterial species at a single concentration of UMP (1 mM) and two concentrations of ATP. The highest concentration of nucleotides (8 mM ATP, 1 mM UMP, and 0.5 mM GTP) was selected arbitrarily to reach the maximal activity for all bacterial species. In Gram-negative organisms (E. coli, S. typhimurium, H. influenzae, and N. meningitidis), the ratio of UMP kinase activity in the presence and absence of GTP was practically independent of the concentration of ATP, whereas in Gram-positive organisms (B. subtilis, S. pneumoniae, S. aureus, and E. faecalis), the activating effect of GTP was much higher at a low concentration of ATP. Further kinetic analysis of UMP kinases from various species clarified the origin of this difference.

Dependence of UMP Kinase Activity on ATP Concentration—E. coli and S. typhimurium UMP kinases were shown to exhibit hyperbolic dependence of activity as a function of ATP concentration in both the absence and presence of GTP (1, 20). The same was true for N. meningitidis UMP kinase. H. influenzae UMP kinase slightly deviates from this rule, as the kinetics with ATP as variable substrate were best fitted by the Hill equation. However, the \( n_i \) values did not exceed 1.30 (Table 4). In the case of UMP kinases from the Gram-positive bacteria, the plot of activity versus the concentration of ATP was clearly sigmoidal in the absence of GTP, with \( n_i \) varying from 1.7 for S. aureus, 2.0 for B. subtilis, and 2.5 for S. pneumoniae. In the presence of GTP, the cooperativity index decreased to almost 1.00, and the \( K_{0.5} \) for ATP decreased by a factor of 3 for S. aureus and 8 for B. subtilis and S. pneumoniae. At saturating concen-
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**TABLE 4**
Kinetic parameters of UMP kinase from three Gram-negative and three Gram-positive organisms with UMP as variable substrate and at a fixed concentration of UMP

The reaction rates were fitted according to the Michaelis-Menten equation \( v = V_m \frac{[ATP]}{([ATP] + K_m)} \) or the Hill equation \( v = V_m \frac{[ATP]^n}{([ATP] + K_m^n)} \), where \( V_m \) is the maximal reaction rate (micromoles/min/mg of protein), \( K_m \) is the Michaelis-Menten constant (millimolar), \( n_H \) is the Hill number, and \( K_m^n \) is the ATP concentration (millimolar) at half-maximal activity.

| Organism                  | Kon or K0.5 | nH | Km or K0.5 | nH | Vm (No GTP) | Km (No GTP) | Km (0.5 mM GTP) | nH (0.5 mM GTP) |
|---------------------------|-------------|----|------------|----|-------------|-------------|----------------|-----------------|
| **E. coli UMP kinase**    |             |    |            |    |             |             |                |                 |
| 0.1 mM UMP                | 66.5 ± 1.5  | 0.23 ± 0.02 | 88.3 ± 1.7 | 0.27 ± 0.02 |
| 1.0 mM UMP                | 42.6 ± 1.3  | 0.15 ± 0.02 | 100.1 ± 1.8 | 0.20 ± 0.02 |
| **N. meningitidis UMP kinase** |         |    |            |    |             |             |                |                 |
| 0.05 mM UMP               | 35.3 ± 1.7  | 2.98 ± 0.41 | 66.0 ± 3.3  | 0.83 ± 0.18 |
| 1.0 mM UMP                | 16.2 ± 0.6  | 3.22 ± 0.36 | 125.0 ± 6.4 | 1.98 ± 0.39 |
| **H. influenzae UMP kinase** |         |    |            |    |             |             |                |                 |
| 0.1 mM UMP                | 56.9 ± 1.7  | 1.57 ± 0.12 | 1.28 ± 0.09 | 1.28 ± 0.09 |
| 1.0 mM UMP                | 30.1 ± 0.9  | 1.62 ± 0.11 | 1.30 ± 0.10 | 1.30 ± 0.10 |
| **B. subtilis UMP kinase** |             |    |            |    |             |             |                |                 |
| 0.1 mM UMP                | 18.2 ± 3.3  | 10.4 ± 2.7 | 1.65 ± 0.2  | 1.65 ± 0.2  |
| 1.0 mM UMP                | 29.5 ± 4.5  | 14.5 ± 1.9 | 2.04 ± 0.32 | 2.04 ± 0.32 |
| **S. pneumoniae UMP kinase** |         |    |            |    |             |             |                |                 |
| 0.1 mM UMP                | 57.2 ± 2.8  | 9.8 ± 0.6  | 2.55 ± 0.30 | 2.55 ± 0.30 |
| 1.0 mM UMP                | 38.6 ± 2.6  | 2.64 ± 0.16 | 1.74 ± 0.28 | 1.74 ± 0.28 |

**TABLE 5**
Kinetic parameters of UMP kinase from three Gram-negative and two Gram-positive organisms with UMP as variable substrate and at a fixed concentration of ATP

The reaction rates were fitted according to the Michaelis-Menten equation or to the equation \( v = V_m \frac{[UMP]}{([UMP] + K_m + [ATP]^n)} \), where \( V_m \) is the maximal reaction rate (micromoles/min/mg of protein), \( K_m \) is the Michaelis-Menten constant (micromolar), and \( K_m^n \) corresponds to the inhibition constant (millimolar).

| Organism                  | Kon or K0.5 | nH | Km or K0.5 | nH | Vm (No GTP) | Km (No GTP) | Km (0.5 mM GTP) | nH (0.5 mM GTP) |
|---------------------------|-------------|----|------------|----|-------------|-------------|----------------|-----------------|
| **E. coli UMP kinase**    |             |    |            |    |             |             |                |                 |
| 0.2 mM ATP                | 45.9 ± 3.6  | 49.2 ± 8.6 | 0.60 ± 0.04 | 46.2 ± 1.4  | 47.0 ± 3.2  |
| 2.0 mM ATP                | 92.5 ± 1.9  | 46.0 ± 4.2 | 0.44 ± 0.03 | 100.3 ± 2.2 | 51.0 ± 4.4  |
| **N. meningitidis UMP kinase** |         |    |            |    |             |             |                |                 |
| 2.0 mM ATP                | 15.7 ± 3.3  | 8.7 ± 4.7 | 0.11 ± 0.05 | 46.4 ± 2.1  | 10.0 ± 2.8  |
| 1.0 mM ATP                | 35.1 ± 5.6  | 15.6 ± 6.7 | 0.23 ± 0.08 | 110.0 ± 2.4 | 57.4 ± 4.7  |
| **H. influenzae UMP kinase** |         |    |            |    |             |             |                |                 |
| 1.0 mM ATP                | 50.8 ± 6.4  | 40.0 ± 10 | 0.31 ± 0.08 | 61.8 ± 0.5  | 40.0 ± 1.0  |
| 12.0 mM ATP               | 109.2 ± 15.5| 100.0 ± 20 | 0.17 ± 0.04 | 75.9 ± 1.0  | 50.0 ± 2.0  |
| **S. pneumoniae UMP kinase** |         |    |            |    |             |             |                |                 |
| 2.0 mM ATP                | 3.2 ± 0.1   | 22.2 ± 2.9 | 4.40 ± 0.98 | 69.3 ± 0.94 | 99.7 ± 5.6  |
| 30.0 mM ATP               | 59.1 ± 1.3  | 150.0 ± 12.2| No inhibition | 80.1 ± 2.4  | 105.6 ± 12.5 |
| **B. subtilis UMP kinase** |             |    |            |    |             |             |                |                 |
| 2.0 mM ATP                | 5.3 ± 0.4   | 10.0 ± 2.1 | 0.66 ± 0.13 | 27.6 ± 0.4  | 27.4 ± 1.9  |
| 30.0 mM ATP               | 41.8 ± 1.0  | 131.0 ± 13.0| No inhibition | 52.0 ± 1.4  | 155.0 ± 14.5 |
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was determined as described for *N. meningitidis* UMP kinase at several constant concentrations of ATP and at variable concentrations of UMP in both the absence and presence of saturating concentrations of GTP. Each individual series was fitted by Equation 2, and the calculated $V_{\text{UMP}}$ (Fig. 2B) values were used for secondary plots with ATP as variable substrate. The resulting constants ($V_{\text{ATP/UMP}}, K_{\text{ATP}},$ and $n_H$) were 68 units/mg of protein, 21 mM, and 1.8 in the absence of GTP and 58 units/mg of protein, 2.9 mM, and 1.1 in its presence, respectively. The kinetics of *S. aureus* UMP kinase with UMP as variable substrate were hyperbolic in both the presence and absence of GTP (data not shown).

**Specificity of Bacterial UMP Kinases for GTP as Activator**—GTP appeared to be the common positive effector for all investigated bacterial UMP kinases (Tables 3–5). The concentration of nucleotide required for half-maximal activation ($K_a$) was independent of the concentration of $Mg^{2+}$ ions. At single concentrations of substrates (2 mM ATP and 1 mM UMP), the $K_a$ of UMP kinases from *E. coli*, *H. influenzae*, *B. subtilis*, and *S. pneumoniae* varied between 70 and 120 μM. *N. meningitidis* UMP kinase exhibited a higher $K_a$ for GTP (~300 μM). When other guanine nucleotides or related compounds such as dGTP, 7-deazaguanosine 5'-triphosphate (dGTP), 3'-anthraniloyl-dGTP, GMP-PNP, ITP, and XTP were tested as activators, a variety of effects were observed (data not shown). Thus, GMP-PNP and dGTP activated all forms of UMP kinases but to a variable extent and affinity compared with GTP. *N. meningitidis* UMP kinase was less sensitive to this activation by GMP-PNP than the other enzymes. GMP was ineffective on *B. subtilis*, *S. pneumoniae*, or *N. meningitidis* UMP kinase, but did activate *H. influenzae* or *E. coli* UMP kinase. 3'-Anthraniloyl-dGTP, a fluorescent analog of dGTP (9), was the strongest activator of *B. subtilis* and *S. pneumoniae* UMP kinases, with a 4-fold lower $K_a$ than for GTP, but was less effective on UMP kinases from the Gram-negative organisms (data not shown).

**Inhibition of UMP Kinase Activity by UTP**—One of the earliest observations regarding *E. coli* UMP kinase that made this enzyme unique among the other NMP kinases was the inhibition by UTP and its reversal by GTP or high concentrations of $MgCl_2$ (1). These results suggested that the true inhibitor of the bacterial enzyme was the magnesium-free UTP and that GTP acted as an antagonist of the former nucleotide. On the other hand, high concentrations of UMP partly protected the enzyme against inhibition by UTP (2). These observations were confirmed with UMP kinase from *N. meningitidis* (Fig. 3A) or *H. influenzae* (data not shown). The $I_{50}$ value for inhibition by magnesium-free UTP of the *N. meningitidis* enzyme at 0.05 mM UMP was 10 μM. A 40-fold increase in UMP concentration shifted the $I_{50}$ to 130 μM magnesium-free UTP. Under the same experimental conditions, inhibition of *B. subtilis* (Fig. 3B) or *S. pneumoniae* (data not shown) UMP kinase by UTP was very little affected by high concentrations of UMP or $Mg^{2+}$ ions.

To better understand these differences between Gram-positive and Gram-negative species, the effect of UTP on individual kinetic constants was further investigated with *E. coli*, *H. influenzae*, and *B. subtilis* UMP kinases. A first series of experiments was conducted at constant concentrations of ATP (around the $K_m$ or $K_{0.5}$ values of individual enzymes) and UTP and at variable concentrations of UMP (Fig. 4, A–C). In the case of *E. coli* and *H. influenzae* UMP kinases, the curves converged at high concentrations of UMP (Fig. 4, A and B), in accordance with the...
observed protective effect against inhibition by UTP of high
UMP concentrations (2). Until a 0.1 mM concentration of UTP,
\(K_m\) for UMP decreased by 34%, whereas their ratio remained almost constant (Fig. 4C). No increase in the apparent \(K_m^{UMP}\) was noticed even at the strongest inhibitory concentrations of UTP. With ATP as variable substrate, inhibition by UTP resulted in an increase in the apparent \(K_m^{ATP}\) or \(K_{0.5}^{ATP}\) for ATP. Thus, in the presence of 0.5 mM UTP, the apparent \(K_m^{ATP}\) or \(K_{0.5}^{ATP}\) for ATP increased by a factor of 4 for \(E. coli\) UMP kinase, a factor of 3.2 for \(H. influenzae\) UMP kinase, and a factor of 3.1 for \(B. subtilis\) UMP kinase (Fig. 4, D–F). GTP in excess of UTP restored the kinetic parameters of bacterial UMP kinases to the values observed in the absence of UTP (Table 6).

Among the UTP analogs tested as inhibitors, dUTP was five times weaker than the corresponding ribonucleotide, whereas TTP was completely ineffective even in the millimolar range. 5-Fluoro-UTP mimicked the effect of UTP with similar (\(E. coli\) and \(H. influenzae\)) or lower (\(B. subtilis\) and \(S. pneumoniae\))
UMP kinases exhibited biphasic kinetics with magnesium-free (9), were not substrates of bacterial UMP kinases. Phosphates (5-bromo-UMP and 5-iodo-UMP), unlike 5-fluoro-UMP (5). It should also be mentioned that the corresponding monophosphates were sensitive to inhibition by 5-bromo-UTP and 5-iodo-UTP (Fig. 5). It should also be mentioned that the corresponding monophosphates (3-bromo-UMP and 5-iodo-UMP), unlike 5-fluoro-UMP (9), were not substrates of bacterial UMP kinases.

**UMP Kinase Activity in the Reverse Reaction**—An essential condition in achieving meaningful quantitative data in the reverse reaction was to maintain "controlled" concentrations of different nucleotide species while varying one single nucleotide. We assumed that ADP, UDP, and GDP form complexes with MgCl₂ with similar values, i.e. 1 mM (18). When GDP substituted efficiently for GTP or GMP-PNP as activator of UMP kinase in the reverse reaction, we used mixtures of these three nucleotides and adjusted the concentration of MgCl₂ according to the relationship indicated under "Experimental Procedures." Both N. meningitidis (Fig. 6A) and B. subtilis (Fig. 6B) UMP kinases exhibited biphasic kinetics with magnesium-free UDP as variable substrate. The apparent values for the nucleotide in the absence of activators were 4 μM (B. subtilis) and 3.4 μM (N. meningitidis). GDP or GMP-PNP increased considerably the reaction rates, reversing almost completely the inhibition caused by excess magnesium-free UDP. As in the forward reaction, GDP or GMP-PNP also increased the apparent values for magnesium-free UDP to 8.5 μM (N. meningitidis) and 24.1 μM (B. subtilis). In the absence of GMP-PNP, the activity of B. subtilis UMP kinase with MgADP as variable substrate was very low even at the highest concentrations of nucleoside diphosphate (Fig. 6C). The major effect of GMP-PNP on the reverse reaction rate was apparently to reverse the inhibition exhibited by both the magnesium-free and magnesium-complexed forms of UDP and consequently to increase the affinity for MgADP.

**Site-directed Mutagenesis Experiments**—Structure analysis of E. coli UMP kinase indicated that the vicinal amino acid residues Thr₁³⁸ and Asn₁₄⁰ are involved in the cross-talk between two adjacent dimers in the hexameric structure (7). The main chain oxygen of Thr₁³⁸ from one subunit is hydrogen-bonded to the side chain nitrogen of Asn₁₄⁰ from the neighboring subunit. The two residues also interact with the base moiety of UMP. As expected, the T138A and N140A variants of E. coli UMP kinase exhibited a much lower thermodynamic stability than the reference protein (7). Substituting Thr₁³⁸, the side chain of which is hydrogen-bonded to uracil, results in a 4-fold higher Kₘ for UMP. In contrast, the Kₘ is not altered by the N140A substitution, as this residue binds uracil only through its main chain carbonyl. The two single residue mutations induce a moderate loss of sensitivity to inhibition by UTP (7). However, the cooperativity of this inhibition appears to be significantly altered. Thus, the cooperativity index of the N140A variant of E. coli UMP kinase declined to 1.5, and the Kₘ increased to 300 μM. As in the case of the reference enzyme, GTP restored the kinetic constants of the UTP-inhibited N140A variant to the values observed in the absence of UTP (Table 6).

Because Thr₁³⁸ and Asn₁₄⁰ of E. coli UMP kinase are conserved as Thr₁³⁵ and Asn₁₃⁷ in the B. subtilis enzyme, we investigated the kinetic properties of the similar variants obtained by site-directed mutagenesis experiments. All three modified variants (T135A, N137A, and T135A/N137A) of B. subtilis UMP kinase exhibited Tₘ values 10 °C lower than that of the wild-type protein. The double mutant T135A/N137A was also the most affected in its stability because, upon dilution in 50 mM Tris-HCl (pH 7.4), it was irreversibly inactivated within several hours. The major kinetic changes (Table 7) are the following: (a) loss of cooperativity with ATP as variable substrate (all modified variants of B. subtilis UMP kinase exhibited hyperbolic dependence of activity in either the absence or presence of GTP); (b) significant increase in the Kₘ for UMP of the T135A variant compared with that of the wild-type enzyme or the N137A variant; and (c) continued sensitivity of both T135A and N137A variants of B. subtilis UMP kinase to activation by GTP, with a 3-fold increase in the Kₘ for activator compared with that of the wild-type enzyme.

### TABLE 6

Reversal by GTP of inhibition by UTP of *E. coli* UMP kinase

| Effector (0.5 mM) | Constant ATP (0.2 mM), variable UMP | Constant UMP (0.3 mM), variable ATP |
|------------------|-------------------------------------|-------------------------------------|
|                  | Constant UTP (0.2 mM), variable UTP | Constant UTP (0.3 mM), variable ATP |
|                  | Vₘ | Kᵤ¹ₒ | Vₘ | Kᵤ¹ₒ |
| Reference (D159N) | 46.1 | 50.0 | 62.4 | 0.21 |
| GTP              | 51.9 | 59.3 | 99.6 | 0.24 |
| UTP              | 36.8 | 1600 | 23.8 | 0.77 |
| GTP + UTP        | 46.6 | 207   | 91.7 | 0.26 |
| N140A variant    | 48.9 | 50.0 | 81.9 | 0.25 |
| GTP              | 58.2 | 73.9 | 110.5 | 0.21 |
| UTP              | 30.4 | 181.2 | 78.3 | 0.53 |
| GTP + UTP        | 55.7 | 108.6 | 98.2 | 0.23 |

**FIGURE 5.** Comparative inhibitory effects of UTP and its 5-halogenated analogs on *H. influenzae* (A) and *B. subtilis* (B) UMP kinases at constant concentrations of ATP (2 (A) and 15 (B) mM) and UMP (0.1 (A) and 0.3 (B) mM). UTP; 5-fluoro-UTP; 5-bromo-UTP; 5-iodo-UTP.
zymes so far described in either prokaryotic or eukaryotic organisms. (i) The primary and three-dimensional structures of bacterial UMP kinases are divergent from those of the other NMP kinases studied and are related to those of the carbamate and N-acetylglutamate kinases (1, 3). (ii) Bacterial UMP kinases are oligomers submitted to a complex control of activity by GTP and UTP. (iii) The membrane proximity of UMP kinases from *E. coli* (21) and *B. subtilis* (22) and most probably from all other bacterial species suggests a specific role of these enzymes in the synthesis of membrane or cell wall constituents.

The cooperative kinetics with respect to ATP of UMP kinase from *S. pneumoniae* (10), a Gram-positive organism, shed new light on this family of catalysts and prompted us to explore or re-examine other UMP kinases from either Gram-positive and Gram-negative bacteria. Our results show that bacterial UMP kinases can indeed be classified in two subfamilies with significantly different regulatory mechanisms. This is not an unprecedented case as, for instance, *E. coli* aspartate transcarbamoylase, the paradigm of allosteric enzymes, exhibits both homotropic and heterotropic interactions (23), whereas *B. subtilis* aspartate transcarbamoylase, a homotrimer, lacks both homotropic and heterotropic interactions (24). Finding the structural basis of these differences in UMP kinases and deciphering the mechanism of regulation are challenging issues. For this purpose, a selection of several representative UMP kinases, some belonging also to pathogenic strains for humans, was a necessary step.

**Common Properties of UMP Kinases from Gram-positive and Gram-negative Bacteria**—Despite the diversity of responses to nucleotides acting as substrates or effectors, the UMP kinases from Gram-negative and Gram-positive bacteria share several common traits. (i) GTP is the common positive effector for all explored enzymes. It reverses the inhibition of excess UMP (forward) or UDP (reverse) and increases the affinity for ATP (forward) or ADP (reverse). (ii) UTP has an opposite effect by decreasing the affinity for ATP/ADP. Whereas in Gram-positive organisms, inhibition by UTP is independent of Mg$^{2+}$/H$^{10}$ ions, in Gram-negative organisms, inhibition by UTP occurs only via magnesium-free nucleotide.

The inhibition caused by excess UMP is variable from one enzyme to another and might depend on pH, the concentration of cosubstrate, or the presence of GTP. At pH 6, inhibition by excess UMP was less apparent or abolished for most examined UMP kinases. At saturating concentrations of ATP, *B. subtilis* UMP kinase was insensitive to inhibition by excess UMP, in contrast to *E. coli* or *H. influenzae* UMP kinase. In all cases, GTP reversed inhibition by excess UMP. Because inhibition by excess nucleoside monophosphate has also been observed with other NMP kinases such as *E. coli* adenylyl kinase (25, 26) and CMP kinase (27) and yeast GMP kinase (28), several common causes might be invoked to explain this phenomenon. Binding of UMP to the MgATP site is excluded, as inhibition is not competitive with MgATP. Binding of UMP to the allosteric site also seems less probable, as isothermal calorimetry showed that

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*Experimental Procedures.* The activators used were GDP (A) and GMP-PNP (B and C). ■, no activator; ●, 0.2 mM activator; ▲, 1 mM activator.
TABLE 7
Kinetic parameters of three modified forms of B. subtilis UMP kinase obtained by site-directed mutagenesis

| UMP as fixed substrate (1 mM), no GTP | Wild-type | T135A | N137A | T135A/N137A |
|-------------------------------------|-----------|-------|-------|-------------|
| Vₘₜ (units/mg)                     | 29.5 ± 4.5| 4.9 ± 0.2| 3.0 ± 0.3| 5.4 ± 1.5 |
| Kₘₜ (mM)                          | 1.4 ± 0.9 | 9.5 ± 0.8 | 10.5 ± 2.1 | 10.0 ± 1.3 |
| nₜ                                  | 2.04 ± 0.31| No cooperativity | No cooperativity | No cooperativity |

| UMP as fixed substrate (1 mM), 0.5 mM GTP* | Wild-type | T135A | N137A | T135A/N137A |
|------------------------------------------|-----------|-------|-------|-------------|
| Vₘₜ (units/mg)                        | 34.0 ± 0.83| 19.0 ± 0.6| 14.7 ± 0.4| 11.3 ± 0.5 |
| Kₘₜ (mM)                             | 1.79 ± 0.11| 1.20 ± 0.2| 1.0 ± 0.1| 2.2 ± 0.4 |
| nₜ                                    | 1.14 ± 0.07| No cooperativity | No cooperativity | No cooperativity |

| ATP as fixed substrate (2 mM), no GTP | Wild-type | T135A | N137A | T135A/N137A |
|--------------------------------------|-----------|-------|-------|-------------|
| Vₘₜ (units/mg)                     | 5.3 ± 0.4 | 0.88 ± 0.035 | 0.65 ± 0.023 | 1.09 ± 0.03 |
| Kₘₜ (mM)                          | 10.0 ± 2.1 | 47.5 ± 8.1 | 12.7 ± 1.9 | 53.1 ± 6.5 |
| Kₙ (mM)                           | 0.66 ± 0.13| No inhibition | No inhibition | No inhibition |

| ATP as fixed substrate (2 mM), 0.5 mM GTP* | Wild-type | T135A | N137A | T135A/N137A |
|------------------------------------------|-----------|-------|-------|-------------|
| Vₘₜ (units/mg)                     | 27.6 ± 0.4 | 9.52 ± 0.11 | 6.75 ± 0.12 | 12.5 ± 0.33 |
| Kₘₜ (mM)                          | 27.4 ± 1.9 | 80.7 ± 4.1 | 17.8 ± 1.6 | 143.0 ± 15.0 |
| Kₙ (mM)                           | No inhibition | No inhibition | No inhibition | No inhibition |

As GTP was at subsaturating concentrations in the case of variants obtained by site-directed mutagenesis, the corresponding Vₘₜ values were underestimated with respect to the wild-type protein.

UMP binds to a single site of E. coli and H. influenzae UMP kinases. The most probable explanation would be the occurrence of an abortive UMP kinase-MgADP-UMP complex, which slows down the release of MgADP. Whatever the true explanation, inhibition by excess UMP of the bacterial UMP kinases also depends on their quaternary structure as demonstrated by site-directed mutagenesis experiments with E. coli (7) and B. subtilis (this study) UMP kinases.

Another property common to various UMP kinases (E. coli appears to be an exception) is that activation by GTP results also in a decrease in the Kₘₜ or Kₙ for ATP. In other words, the positive effector acts simultaneously on the kinetic constants of both nucleotide substrates, irrespective of the cooperativity or noncooperativity existing toward the phosphate donor. As a corollary, the complex kinetic effects exhibited by the negative effector (UTP), i.e. a significant increase in the apparent Kₘₜ or Kₙ for ATP and a change in the apparent Kₘₜ for UMP, were not surprising. The fact that the apparent Kₘₜ for UMP of E. coli and H. influenzae UMP kinases increased dramatically at concentrations of ATP above 0.1 mM is related to the cooperative binding of UTP to its site, which is consistent with the fluorescence properties of the E. coli UMP kinase-UTP complex (1, 6).

Differences between UMP Kinases from Gram-positive and Gram-negative Organisms—The major difference between UMP kinases from Gram-negative and Gram-positive organisms is the lack of cooperativity with ATP in the former organisms. Although, with H. influenzae UMP kinase, the best fittings of reaction rates with ATP as variable substrate were obtained using the Hill equation, the nₜ values never exceeded 1.3. On the other hand, the activation of Gram-negative N. meningitidis UMP kinase by GTP is a combination of several effects: enhancement of Vₘₜ, increase in affinity for ATP, and reversal of inhibition by excess UMP. In this respect, it is worth mentioning that cooperativity in allosteric enzymes is mediated via changes in affinity for substrates (K systems) or via changes in the maximal velocity (V systems) (29). UMP kinases from Gram-positive organisms belong clearly to the K systems, i.e.

Both T and R states have the same Vₘₜ values, but different affinities for ATP. In the absence of effectors, the binding of ATP is cooperative, and the positive homotropic interaction is lowered in the presence of GTP or its analogs (10). A factor that might contribute to the cooperativity with ATP of UMP kinase from Gram-positive bacteria might be the dissociation of active hexamers into lower molecular mass oligomers. Such reversible dissociation of hexamers was never observed with E. coli or H. influenzae UMP kinase.

Another major difference between UMP kinases from Gram-positive and Gram-negative organisms is related to their sensitivity to inhibition by UTP and their halogenated analogs. In Gram-positive bacteria, inhibition by UTP is not sensitive to high concentrations of Mg²⁺ or UMP, whereas in Gram-negative organisms, inhibition by UTP is reversed by high concentrations of divalent ion or UMP. On the other hand, the halogen-substituted UTP analogs demonstrate strikingly different effects on UMP kinases from Gram-positive and Gram-negative organisms, suggesting that they interact with different sites in the UMP kinases from these two families of bacteria.

Identity of the Effector-binding Site(s) and Mechanism of Regulation of Bacterial UMP Kinases—The existence of either a unique or two distinct binding sites for GTP and UTP was raised at the very beginning of our study of bacterial UMP kinases (1). From the kinetic experiments described in this work, corroborated by previous spectroscopic and site-directed mutagenesis experiments (2, 6) and the x-ray analysis of E. coli UMP kinase in complex with GTP, we can confidently assume that each subunit of bacterial UMP kinase, irrespective of its origin, has three distinct nucleotide-binding sites. The fundamental difference between Gram-positive and Gram-negative organisms is related to the occupancy of these sites by nucleotides and their corresponding analogs. Two of these sites conserved throughout different bacterial species belong to the catalytic center. They interact with ATP or ADP, either as magnesium complexes or magnesium-free nucleotides, and with UMP or UDP, only as magnesium-free nucleotides. The

3 C. T. Craescu, P. Christova, and O. Popescu, unpublished data.

4 P. Briozzo and P. Meyer, unpublished data.
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third site, less conserved than the previous ones, interacts primarily with GTP, either as magnesium-free or magnesium-complexed species, and in, some particular cases, with GDP, GMP, and even cGMP or guanosine (1). The GTP-binding site, located at the interface of two vicinal monomers, is most probably common to all bacterial species sensitive to activation by guanine nucleotides and/or related analogs. Comparison of a E. coli UMP kinase–GTP complex with other structurally known bacterial UMP kinases (H. influenzae and S. pyogenes) indicates an identical fold and distribution of amino acid residues critical for binding of this effector. 4 In Gram-positive organisms, the GTP-binding site corresponds to the single allosteric site, commonly described for the vast majority of regulatory proteins. It can therefore be designed as the “GTP/UTP site” or “effector site.” Binding of effectors to this site shifts regulatory proteins. It can therefore be designed as the “GTP/UTP site” or “effector site.”

Physiological Relevance of Regulatory Effects of GTP and UTP on Bacterial UMP Kinases—UMP kinase is the first of the three enzymes involved in the conversion of UMP to UTP and CTP, the last two being NDP kinase and CTP synthetase. Both UMP kinase and CTP synthetase are oligomeric proteins positively regulated by GTP and use nucleotides as substrates (1, 30–32). The structural and functional complexity of these two bacterial enzymes and the inhibition of their activity by the end products UTP and CTP, respectively, indicate that they might be submitted also in vivo to a closely similar control of activity by nucleotides and Mg2+ ions. Assuming that, in bacteria such as E. coli and B. subtilis, the concentrations of ATP, GTP, UTP, and UMP oscillate around 2–3 mM (ATP), 0.8–1.2 mM (GTP and UTP), and 0.050–0.1 mM (UMP) (33, 34) and that the concentration of soluble Mg2+ is ∼15 mM (35, 36), we might speculate about a role of these nucleotides in modulating the activity of individual UMP kinases. In the case of Gram-positive bacteria, the UMP kinases of which exhibit low affinity for ATP in the absence of GTP, it is obvious that the latter nucleotide is a major participant besides the two substrates. Once the UTP pool is saturated, it competes with GTP for the allosteric site, lowering UMP kinase activity. In Gram-negative organisms, the situation appears to be different. Thus, in bacteria such as H. influenzae and N. meningitidis, the Kₘ for ATP of the corresponding UMP kinases is of the same order of magnitude as the cellular concentration of this nucleotide. Consequently, the cooperative inhibition by magnesium-free UTP at >10 μM might be physiologically relevant. The role of GTP would be rather compensating as an “antagonist of the inhibitor.” E. coli has apparently the most “buffered” UMP kinase system, with the enzyme operating always under saturating concentrations of ATP (10 times the corresponding Kₘ). One of the future tasks in addition to the precise identification of the allosteric site of UMP kinases from Gram-positive organisms will be to determine the in vivo coupling of the UMP kinase and CTP synthetase activities, as much as both enzymes represent valuable targets for antibacterial agents.

Acknowledgments—We thank Pierre Briozzo (Institut National de la Recherche Agronomique Paris-Grignon) for constructive criticism and for providing information ahead of publication, Yves Janin for carefully reading this manuscript and helpful suggestions, Cristina Gagyi and Ovidiu Sirbu for participation in the preliminary experiments, and Jean-Claude Rousselle and Pascal Lenormand for mass spectrometry measurements.

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