Escherichia coli UMP-kinase, a Member of the Aspartokinase Family, Is a Hexamer Regulated by Guanine Nucleotides and UTP†

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Received August 22, 1994; Revised Manuscript Received January 20, 1995⊗

ABSTRACT: The *pyrH* gene, encoding UMP-kinase from *Escherichia coli*, was cloned using as a genetic probe the property of the *carAB* operon to be controlled for its expression by the concentration of cytoplasmic UTP. The open reading frame of the *pyrH* gene of 723 bp was found to be identical to that of the *smbA* gene [Yamanaka, K., et al. (1992) *J. Bacteriol. 174*, 7517−7526], previously described as being involved in chromosome partitioning in *E. coli*. The bacterial UMP-kinase did not display significant sequence similarity to known nucleoside monophosphate kinases. On the contrary, it exhibited similarity with three families of enzymes including aspartokinases, glutamate kinases, and *Pseudomonas aeruginosa* carbamate kinase. UMP-kinase overproduced in *E. coli* was purified to homogeneity and analyzed for its structural and catalytic properties. The protein consists of six identical subunits, each of 240 amino acid residues (the N-terminal methionine residue is missing in the expressed protein). Upon excitation at 295 nm, the bacterial enzyme exhibits a fluorescence emission spectrum with maximum at 332 nm which indicates that the single tryptophan residue of the protein (Trp119) is located in a hydrophobic environment. Like other enzymes involved in the *de novo* synthesis of pyrimidine nucleotides, UMP-kinase of *E. coli* is subject to regulation by nucleotides: GTP is an allosteric activator, whereas UTP serves as an allosteric inhibitor. UTP and UDP, but none of the other nucleotides tested such as GTP, ATP, and UMP, enhanced the fluorescence of the protein. The sigmoidal shape of the dose−response curve indicated cooperativity in binding of UTP and UDP. A UMP-kinase mutant (D201N) recognized earlier as responsible for altered morphological phenotype in *E. coli* (Yamanaka et al., 1992) was analyzed for its stability and kinetic properties. The protein exhibited 10% of the activity of the wild-type enzyme and had altered stability and regulatory properties. This favors the hypothesis that UMP-kinase, i.e., SmbA protein, participates only indirectly in cell division.

Nucleotides are at the core of living processes. The enzymes responsible for the synthesis, degradation, or interconversion of nucleotides have been used for many years as targets for antiviral, antibacterial, or antiparasitic drugs (Suhadolnik, 1970; Bloch, 1975; Blackburn & Gait, 1990). Among these enzymes nucleoside monophosphate kinases (adenylate kinase, guanylate kinase, uridylate kinase) form a homogeneous family of catalysts thought to derive from a common ancestor. In spite of their specificity for individual purine or pyrimidine monophosphates, they share substantial sequence similarities and related three-dimensional structure (Müller & Schulz, 1992; Stehle & Schulz, 1992; Bârzu & Gilles, 1993; Müller-Dieckman & Schulz, 1994). However, significant variations in structure and catalytic properties among nucleoside monophosphate kinases from various species have been identified in the last years. Thus, adenylate kinases from bacteria, yeast, and mitochondria differ from the mammalian cytosolic variant by an insertion of 25 amino acid residues that are missing in the latter protein (Schulz et al., 1986; Schulz, 1987). Moreover, adenylate kinases from Gram-positive bacteria, but not from Gram-negative organisms, were shown to bind one atom of zinc per molecule (Glaser et al., 1992). This property was correlated with the presence of three or four cysteine residues in the inserted sequence (Gilles et al., 1994; Perrier et al., 1994).

These recent and surprising observations prompted us to undertake a systematic analysis of nucleoside monophosphate kinases other than adenylate kinase. Our aim was to correlate differences in structure with substrate specificity, stability, regulatory properties, and capability of organisms to adapt to environmental conditions. Preliminary biochemical investigations on UMP-kinase from *Escherichia coli* and other enteric bacteria (*Salmonella typhimurium*, *Enterobacter cloacae*, *Yersinia enterocolitica*) revealed unusual properties, never described before, such as (i) stability at temperatures up to 65 °C in the absence of protective agents; (ii) molecular masses greater than 140 kDa, as determined by gel permeation chromatography; and (iii) activation by GTP and inhibition by UTP. Since the *pyrH* gene of *E. coli* was shown to encode a protein (Smallshaw & Kelln, 1992) which did not display similarities with known UMP-kinases (Liljelund et al., 1989; Wiesmüller et al, 1990), we hypothesized that the bacterial UMP-kinase might deviate from the

† This work was supported by grants from the Centre National de la Recherche Scientifique (URA 1119) and from the Institut Pasteur.

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⊗ Abstract published in *Advance ACS Abstracts,* April 1, 1995.

0006-2960/95/0434-5066$09.00/0

nucleoside monophosphate kinase paradigm. This was indeed the case, as shown in the present work in which we characterized the protein after overexpression of the corresponding gene. Like other *E. coli* enzymes involved in the *de novo* synthesis of UTP and CTP, UMP-kinase has an oligomeric structure and is subject to complex regulatory control by GTP and UTP. Moreover, the sequence of the *pyrH* gene was found to be identical to that of the *smbA* gene, previously described as being involved in chromosome partitioning in *E. coli* (Yamanaka et al., 1992), which raises questions about the function of UTP or of the protein itself in this important cellular event.

EXPERIMENTAL PROCEDURES

Chemicals. Adenine nucleotides, restriction enzymes, T4 DNA ligase, and coupling enzymes were from Boehringer Mannheim. T7 DNA polymerase and the four deoxynucleoside triphosphates used in sequencing reactions were from Pharmacia. Dial-GTP[1] was a product of Sigma. Oligonucleotides were synthesized according to the phosphoramidate method using a commercial DNA synthesizer (Cyclone TM Biosearch). NDP-kinase from *Dictyostelium discoideum* (2000 units/mg of protein) was kindly provided by M. Véron.

Bacterial Strains, Plasmids, Growth Conditions and DNA Manipulations. E. coli strain 14-40-42 [F$^-$, *araD139*, Δ(*lacIPOZYA*)*U169*, *rpsL*, *thi*, *pyrH42*, *car14*(::Mud*lac*Ap^R)] with a *carAB*::*lacZ* fusion, a kind gift of N. Glansdorff, was used as a recipient for an *E. coli* genomic library constructed in pSU21 (Martinez et al., 1988). The XL1 Blue strain (Bullock et al., 1987) was used for DNA sequencing. Uracil-containing single-stranded DNA was produced by the *ung dut E. coli* strain RZ1032 (Kunkel, 1985). Strain BLI5 used for overexpression of the *pyrH* gene was derived from BL21 (DE3) (Novagen, Inc.). This strain expresses the *lacI* gene on plasmid pDIA17 and the T7 RNA polymerase gene on the chromosome. Cultures were performed in 2YT medium (Sambrook et al., 1989) supplemented with 100 μg/mL ampicillin and 30 μg/mL chloramphenicol. When OD_{600} reached 1.5, 1 mM IPTG was added to the medium. Bacteria were harvested by centrifugation 3 h after induction.

Site-directed mutagenesis was performed on the single DNA strand form of phagemid pDIA5418 grown in strain RZ1032 in the presence of plasmid pDIA17 and the helper phage M13K07 (Blondel & Thillet, 1991). The Asp (GAC) codon at position 201 was modified to a Asn (AAC) codon using the oligonucleotide 5′GGCCGCCAGGTTCATGACTTT3′. The absence of unwanted mutations during the site-directed mutagenesis was verified by the dideoxynucleotide sequencing method (Sanger et al., 1977).

Analytical Procedures. UMP-kinase activity was determined in both directions at 30 °C and 334 nm using coupled spectrophotometric assays (0.5 mL final volume) on an Eppendorf PCP6121 photometer (Blondin et al., 1994). The reaction medium contained either 50 mM Tris-HCl (pH 7.4), 50 mM KCl, 2 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 0.2 mM NADH, different concentrations of ATP and UMP, and 2 units each of lactate dehydrogenase, pyruvate kinase, and nucleoside diphosphate kinase (forward reaction), or 50 mM Tris-HCl (pH 7.4), 50 mM KCl, 2 mM $MgCl_2$, 1 mM glucose, 0.4 mM $NADP^+$, different concentrations of ADP and UDP, and 2 units each of hexokinase and glucose-6-phosphate dehydrogenase (reverse reaction). The reaction was started with UMP-kinase. One unit of UMP-kinase corresponds to 1 μmol of product formed per minute.

Protein concentration was measured according to Bradford (1976) or by amino acid analysis on a Beckman system 6300 high-performance analyzer after 6 N HCl hydrolysis for 22 h at 110 °C. SDS−polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). The protein band from SDS−PAGE was electroblotted onto a Problott membrane filter (Applied Biosystems) and detected by staining with Coomassie blue. The N-terminal amino acid sequence of the protein from the excised band was determined by a protein sequencer (Applied Biosystems Inc.).

Fluorescence measurements were done with a Perkin Elmer LS-5B luminescence spectrometer thermostated at 25 °C using 1 × 1 cm UV-grade quartz cuvettes (sample volume, 2 mL). Emission spectra of UMP-kinase (λ_{exc} = 295 nm; band width = 5 nm) were recorded from 305 to 400 nm. Titration of UMP-kinase with nucleotides (2% maximal dilution of the sample) was determined by fluorescence enhancement at 330 nm. One data point corresponds to fluorescence intensities integrated over a total time of 8 s.

Equilibrium sedimentation was performed at 20 °C on a Beckman Optima XLA ultracentrifuge using a AN 60 Ti rotor and a cell with a 12-mm optical path length. Samples (100 μl) in 0.1 M borate buffer (pH 8.6) at approximately 0.5 mg of protein/mL were centrifuged at 6000 and 9000 rpm. Radial scans of absorbance at 280 nm were taken at 2-h intervals. Equilibrium was achieved after 18 h centrifugation. Data were analyzed by using the programs XLAEQ and EQASSOC supplied by Beckman. The partial specific volume of the protein (0.749 cm^3/g) was calculated from its sequence according to Perkins (1986).

RESULTS

Cloning and Sequencing of the pyrH Gene and "in Silico" Analysis of the Gene Product. Since UTP is indispensable for cell survival, it was likely that mutants harboring the *pyrH* defect would correspond to a gene specifying a protein with some residual function. We used as a genetic screen the property of the *carAB* operon expression to be controlled by the concentration of cytoplasmic UTP. Using a *carAB*::*lacZ* fusion expressed at a high level in a *pyrH* background, we screened an *E. coli* library for a low level of β-galactosidase expression. Clones displaying high UMP-kinase activity *in vitro* were kept for further study. Subcloning and sequencing experiments led to the identification of an open reading frame which was found to be identical to the *smbA* gene (Figure 1A). This sequence did not display any significant similarity to known nucleoside monophosphate kinases. To better characterize the gene product, we undertook some computer-assisted analysis ("in silico" analysis).

Sequence comparison was performed as follows. A first scan of the protein data libraries present at the NCBI

[1] Abbreviations: Ap_5A, P^1,P^5-di(adenosine-5′)pentaphosphate; Ap_5U, P^1-(adenosine-5′)-P^5-(uridine-5′)pentaphosphate; dial-GTP, dialdehyde-GTP, a ribose ring opened derivative of GTP; dial-UTP, dialdehyde-UTP, a ribose ring opened derivative of UTP; GMP-PNP, guanylyl imidodiphosphate; IPTG, isopropyl β-D-thiogalactoside; NDP-kinase, nucleoside diphosphate kinase (EC 2.7.4.6); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

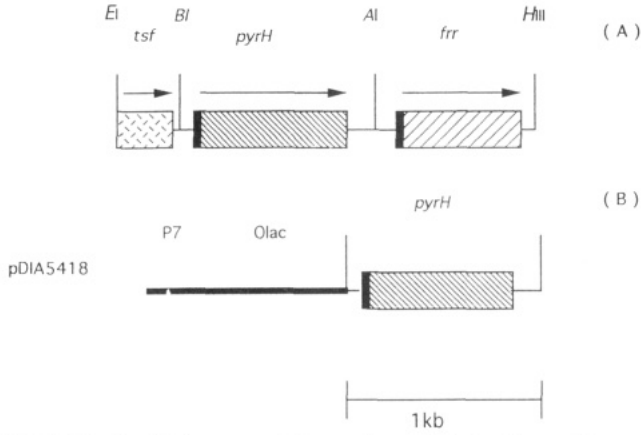

FIGURE 1: Restriction map of the region containing the *pyrH* gene of *Escherichia coli*. (A) Relevant restriction sites with respect to the 2.0-kb *Eco*RI−*Hin*dIII fragment are indicated. This whole region of DNA was sequenced. The arrows indicate the limits of the structural genes contained (*pyrH*, *frr*, and the end of *tsf*) and the direction of transcription. Each gene is represented with its promoter (dark boxes). (B) Subcloning of the 1.0-kb Bsu361-*Ava*I fragment containing only the *pyrH* gene in the expression vector pET22b represented by the thick line. This vector contains a strong promoter specifically recognized by the T7 polymerase, and the *lac* operator. This construction is called pDIA5418. *E*I, *Eco*RI; *B*I, *Bsu*361; *A*I, *Ava*I; *H*III, *Hin*dIII.

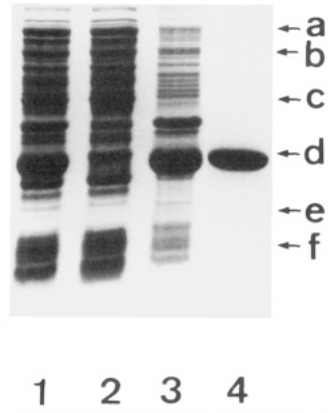

FIGURE 2: SDS−PAGE (12.5%) analysis of *E. coli* UMP-kinase. Lane 1, sonicated bacteria (30 μg of protein); lane 2, supernatant after centrifugation of sonicated bacteria (28 μg of protein); lane 3, pellet resuspended in 50 mM Tris-HCl (pH 7.4) after centrifugation of sonicated bacteria (27 μg of protein); lane 4, pure UMP-kinase (18 μg of protein). Arrows on the right side indicate the molecular mass markers: (a) phosphorylase *b* (94 000), (b) bovine serum albumin (67 000), (c) ovalbumin (43 000), (d) carbonic anhydrase (30 000), (e) soybean trypsin inhibitor (20 300), (f) lysozyme (14 400).

was performed using the BLAST program (Altschul et al., 1990), and sequences displaying a score higher than 65, with a Poisson distribution probability lower than 1, were retained for further study. The sequence of yeast aspartokinase displayed a score of 92, with a Poisson probability of 0.00049, the 145−194 region of UMP-kinase displaying 40% identities and 56% conservative replacements. In the list of 17 sequences displaying similarities, several other kinases were also present. The region of similarity encompassed in all cases the same segment (145−194 or larger) of the UMP-kinase. A further indication that this was significant was the observation that conserved residues were present at similar locations in the sequences. It is well known that many false positives or false negatives are linked to the usage of the BLAST program. False positives mainly stem from sequences having repeated segments, in particular containing Pro, Gly, Ala, or Arg residues. This is not what we observe in our case. False negatives come from the fact that a single matrix of equivalence is used for all positions in the sequence, which is certainly inadequate to represent reality. A weaker similarity with glutamate kinases, enzymes involved in the biosynthesis of proline, was also found. For this reason we constructed from the alignment provided by the BLAST search a consensus matrix for the conserved regions. This permitted us to search the SwissProt library (release 39) for other sequences which might have escaped our attention, using the SmartCons program (A. Viari, unpublished). SmartCons uses a matrix of equivalent residues for each position, built up from a given alignment, for scanning protein data bases. In this way was discovered the similarity with *Pseudomonas aeruginosa* carbamate kinase.

A further confirmation that the sequence thus identified was significant came from the use of the Smith and Waterman (1981) approach implemented at the EMBL facilities on a highly parallel multiprocessor computer [program Blitz: Sturrock and Collins (1993), version 1.5; e-mail to Blitz@embl-heidelberg.de]. Combining both approaches, we identified in the C-terminal half of UMP-kinase a gap-free region situated between amino acid residues 142 and 194 displaying significant similarities with aspartokinases. By the introduction of gaps, this region was expanded to encompass amino acids 134−233, i.e., the carboxy-terminal half of the molecule. This overall similarity seems to indicate that the corresponding region might belong to the catalytic site of the UMP-kinase. Exploration of the amino-terminal half of UMP-kinase revealed in one case a short region of similarity with a human cDNA, coding for a protein whose function is unknown.

Purification and Molecular Characterization of UMP-kinase. The shortest fragment cloned into vector pET22b expressed UMP-kinase to a level about 500 times that found in the wild-type *E. coli* (Figure 1B). The overproduced UMP-kinase was recovered in the pellet fraction after *E. coli* breakage in 50 mM Tris buffer at pH 7.4. The protein, exhibiting low solubility at neutral pH, was easily solubilized from the pellet with 100 mM borate (pH 8.5) and reprecipitated by overnight dialysis against 50 mM Tris-HCl (pH 7.4). Several washing/centrifugation cycles in 50 mM Tris-HCl buffer (pH 7.4) allowed recovery of pure UMP-kinase (Figure 2). Stored at room temperature for 2 months in 0.1 M borate buffer (pH 8.5), the enzyme conserved 100% of its initial activity.

The first seven N-terminal amino acid residues, Ala-Thr-Asn-Ala-Lys-Pro-Val, corresponded to those deduced from the *smbA*/*pyrH* gene except that the N-terminal methionine residue was missing. The calculated M_r of the protein was 25 802. The molecular mass of UMP-kinase determined by sedimentation equilibrium (156 kDa) indicated that under native conditions the *E. coli* enzyme is a hexamer. The purified recombinant protein was stable against thermal denaturation (Figure 3), although the temperature of half-inactivation was 5 °C lower than that of the protein in crude extracts of wild-type *E. coli*.

Kinetic Properties of UMP-kinase from E. coli. Determination of the reaction rates at variable concentrations of ATP and UMP revealed Michaelis−Menten kinetics with apparent K_m values for ATP (at 1 mM UMP) of 0.12 mM

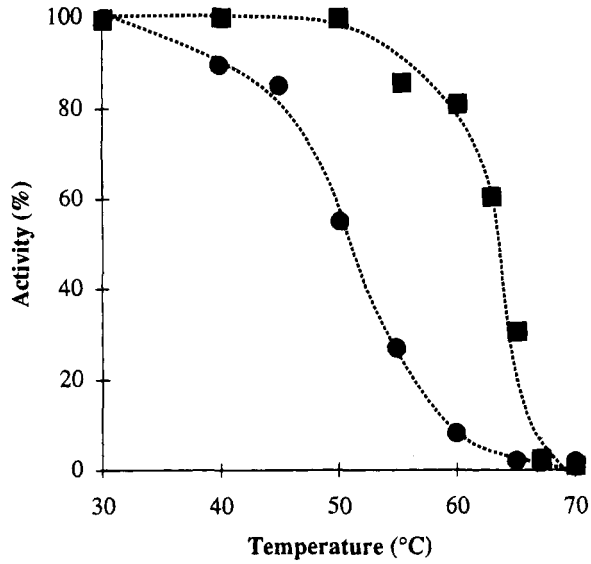

FIGURE 3: Thermal inactivation of wild-type (■) and D201N mutant (●) *E. coli* UMP-kinase. Purified enzyme solutions (0.5 mg of protein/ml) in 0.1 M borate (pH 8.5) were heated for 10 min at various temperatures between 40 and 70 °C, and then residual activity in the forward reaction was determined with 1 mM ATP + 1 mM UMP.

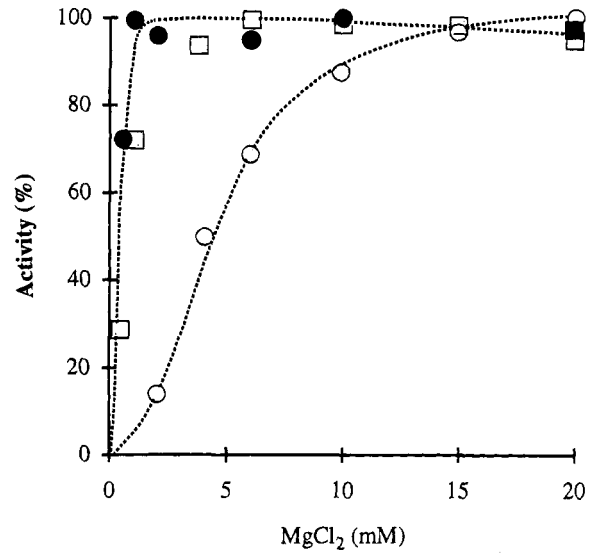

FIGURE 4: Effect of Mg^{2+} concentration on UMP-kinase activity in the forward reaction with ATP, GTP, or UTP as phosphoryl group donor. Enzyme activity in the forward reaction was determined with 1 mM nucleoside triphosphate and 1 mM UMP as described under Experimental Procedures. Various concentrations of $MgCl_2$ were included in the reaction mixture. When GTP or UTP was the phosphate donor, NDP-kinases was omitted from the reaction mixture, and pyruvate kinase was increased to 15 units/sample. 100% corresponds to 50 units/mg of protein (ATP as substrate, ●), 1.8 units/mg of protein (GTP as substrate, □) or 2.2 units/mg of protein (UTP as substrate, ○).

and for UMP (at 1 mM ATP) of 0.05 mM. At UMP concentrations over 0.3 mM the enzyme activity with ATP as cosubstrate declined gradually to reach approximately 65% of its maximum at 1 mM nucleoside monophosphate. None of the other pyrimidine nucleoside monophosphates (dUMP, CMP, dCMP, TMP) acted as substrates or as inhibitors of the *E. coli* enzyme. From all nucleoside triphosphates tested, GTP and UTP showed by far the most interesting effects. Both of them are poor substrates of bacterial enzyme but have different requirements for optimal activity. Thus, over 1 mM $MgCl_2$ the activity of *E. coli* enzyme with ATP and

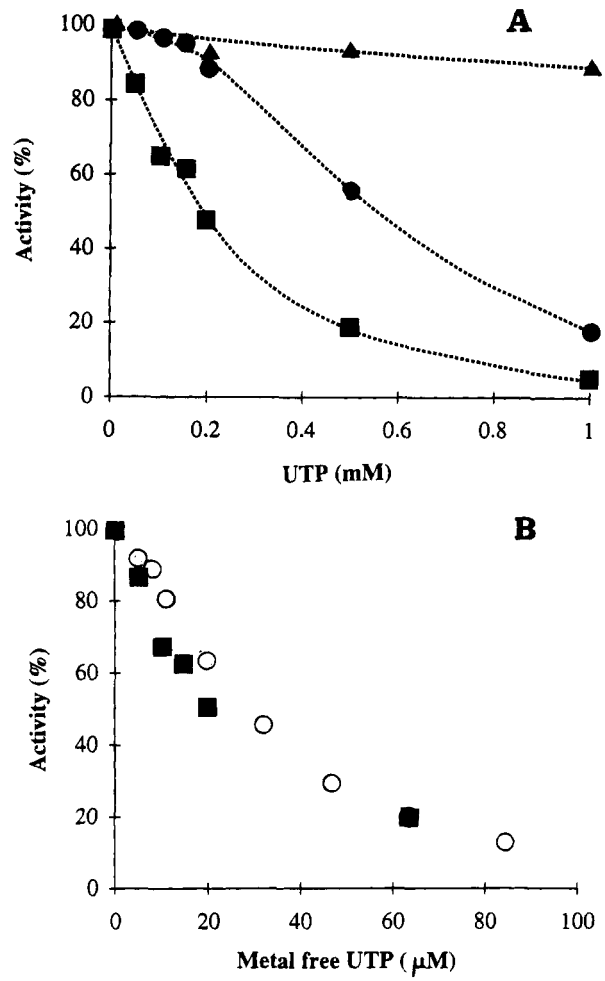

FIGURE 5: Inhibition by UTP of UMP-kinase activity and reversal of its effect by $MgCl_2$ or GMP-PNP. (A) Enzyme activity in the forward reaction (1 mM ATP + 1 mM UMP) supplemented (●) or not (■ , ▲) with 0.5 mM GMP-PNP was determined in the presence of various concentrations of UTP. $MgCl_2$ concentration was either 2 mM (● , ■) or 20 mM (▲). 100% corresponds to 53 units/mg of protein (■), 50 units/mg of protein (▲) or 155 units/mg of protein (●). (B) Enzyme activity shown in Figures 4 (with UTP as phosphoryl donor, ○) and 5A (with ATP as phosphoryl donor, ■) was replotted as a function of metal-free UTP concentration. This last parameter was calculated assuming that the dissociation constants (K_d) of Mg^{2+}−ATP or Mg^{2+}−UTP complexes are identical, and under our experimental conditions, they are approximately 0.1 mM (Bârzu et al., 1976). The 100% activity (i.e., in the absence of metal-free UTP) cannot be determined experimentally when UTP serves also as substrate. This value was calculated by plotting first $1/v$ versus metal-free UTP concentration (Dixon plot).

GTP as phosphoryl donors attained its maximum. At the same concentration of divalent cation the enzyme activity with UTP was negligible compared to that found at 20 mM $MgCl_2$ (Figure 4). The plot of activity with UTP as phosphoryl donor versus $MgCl_2$ concentration showed a sigmoidal shape. Determination of UMP-kinase activity at a single and high concentration of $MgCl_2$ (20 mM) and variable concentrations of UTP showed a slight sigmoidal shape (Hill number between 1.5 and 1.6 and $S_{0.5}$ of 0.3 mM). When UMP-kinase activity was determined in the presence of both ATP (single concentration) and UTP (variable concentrations) and 2 mM $MgCl_2$, the reaction rate decreased much more than expected from a purely competititive effect (I_{50} for UTP was approximately 0.2 mM). At 20 mM $MgCl_2$ the inhibitory effect of UTP was almost completely reversed

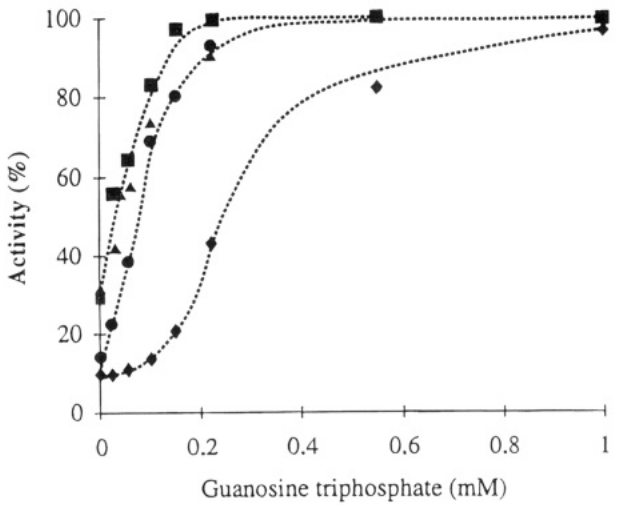

FIGURE 6: Effect of GTP or GMP-PNP on the forward and reverse reactions catalyzed by *E. coli* UMP-kinase. Enzyme activity in the forward reaction was determined with 1 mM ATP + 1 mM UMP as described under Experimental Procedures. Various concentrations of GTP (▲) or GMP-PNP (■) were included in the reaction mixture. 100% corresponds to 160 units/mg of protein. Enzyme activity in the reverse reaction was determined in the presence of 1 mM ADP, 1 mM UDP (♦) or 0.1 mM UDP (●), and various concentrations of GMP-PNP. 100% corresponds to 42 units/mg of protein.

(Figure 5A). These results strongly suggested a dual effect of UTP on UMP-kinase from *E. coli*. First, the Mg^{2+}-UTP complex acts as a substrate (4−5% of activity found with ATP). The metal-free UTP, on the other hand, is a relatively strong inhibitor (I_{50} between 20 and 30 μM) of the bacterial enzyme upon binding to a second, allosteric site (Figure 5B).

The reaction rate of UMP-kinase from *E. coli* with GTP as phosphoryl donor and under standard assay conditions (i.e., 1 mM each of GTP and UMP and 2 mM $MgCl_2$) was 3−5% of that with ATP, and the apparent K_m for GTP was approximately 1 mM. When both ATP and GTP were present, the UMP-kinase activity was increased by a factor of 3 (160 units/mg of protein) as compared to the activity with ATP as sole nucleoside triphosphate substrate. In other words, GTP has also a dual effect, as a poor substrate and as an activator of bacterial UMP-kinase. GMP-PNP, a nonhydrolyzable GTP analog, exhibited effects on the *E. coli* UMP-kinase activity similar to those of the natural nucleotide (Figure 6). On the other hand, GMP-PNP partly reversed the inhibitory effect of UTP on enzyme activity (Figure 5A). dGTP, GMP, cGMP, and even guanosine also activated UMP-kinase, but with lower affinities or extent of activation (data not shown). Dial-GTP, a periodate oxidized derivative of GTP, had a slight stimulatory effect (30% maximal increase in UMP-kinase activity).

The UMP-kinase activity in the reverse reaction, with 1 mM ADP and 1 mM UDP as substrates, was low (3 units/mg of protein). In the presence of GMP-PNP, the activity increased by a factor of 10−16-fold (Figure 6). At lower concentrations of UDP (0.1 mM), the GMP-PNP concentration required for half-maximal activation decreased from 260 to 80 μM. We concluded, therefore, that UDP acts not only as a substrate but also as an inhibitor, much like UTP, i.e., as a metal-free nucleotide. In fact, with an increase in the Mg^{2+} concentration from 2 to 20 mM the reaction rates with 1 mM ADP + 1 mM UDP increased by a factor of 10 (data not shown).

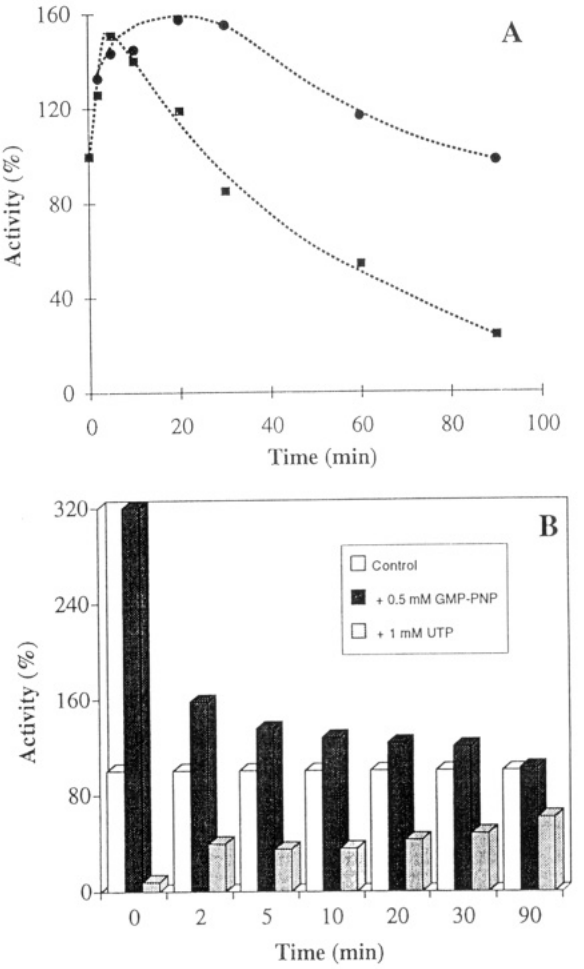

FIGURE 7: Effect of dial-GTP on the activity (A) and the sensitivity of *E. coli* UMP-kinase to activation by GMP-PNP or inhibition by UTP (B). (A) UMP-kinase in 0.1 M borate (pH 8.5) at 1 mg of protein/ml was incubated at room temperature with 5 mM dial-GTP in the presence (●) or absence (■) of 10 mM ATP. At various time intervals samples of protein were diluted 50 times with borate buffer, and then enzyme activity was determined in the forward reaction with 1 mM ATP + 1 mM UMP. (B) Samples of UMP-kinase incubated with dial-GTP in the absence of ATP (squares in panel A) withdrawn at various time intervals were assayed in the forward reaction as in panel A, without or with 0.5 mM GMP-PNP or 1 mM UTP. Activation by GMP-PNP and inhibition by UMP were relative to samples in the absence of effectors.

Ap_5A and Ap_5U, two well-known inhibitors of various forms of adenylate kinase (Lienhard & Secemski, 1973; Feldhaus et al., 1975; Glaser et al., 1992) or UMP-kinase from *Dictyostelium discoideum* (Wiesmüller et al., 1990), were also tested on UMP-kinase from *E. coli*. The I_{50} value of Ap_5U on bacterial enzyme (20 μM) was considerably higher than that found on the enzyme from *D. discoideum* (<0.1 μM) under similar experimental conditions. Ap_5A was even less effective on UMP-kinase from *E. coli* (I_{50} > 0.1 mM).

Covalent Modification of UMP-kinase by Dial-GTP. Since the effects of GTP or GMP-PNP and UTP were mutually exclusive, we examined whether these nucleotides might interact with the same site of the protein. For this purpose UMP-kinase was incubated with dial-GTP at alkaline pH. Covalent attachment of the analog to the protein was expected to "lock" UMP-kinase in a permanently activated form and to render the enzyme insensitive to inhibition by UTP. As shown in Figure 7, dial-GTP has a biphasic effect on UMP-kinase from *E. coli*: activation up to 50% within

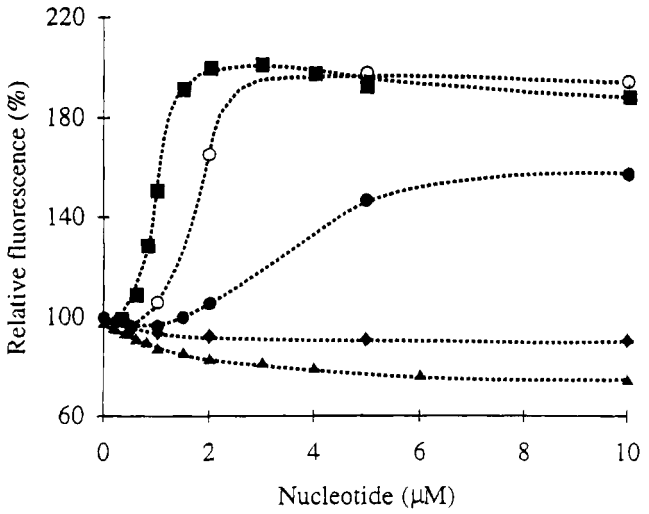

FIGURE 8: Fluorescence analysis of the interaction between nucleotides and native UMP-kinase or dial-GTP-modified enzyme. UMP-kinase (1 μM in terms of monomer) in 50 mM Tris-HCl (pH 7.4) and 100 mM NaCl was titrated at 330 nm (λ_{exc} = 295 nm) with different nucleotides: UTP (■), UDP (○), dUTP (●), and UMP (▲). Dial-GTP-modified UMP-kinase was prepared as follows: enzyme in 0.1 M borate (pH 8.5) at 1 mg of protein/ml was incubated at room temperature for 90 min with 5 mM dial-GTP and 10 mM ATP. Then, the nucleotides were removed by chromatography onto a Sephadex G-25 column equilibrated with 0.1 M borate (pH 8.5). (♦) Titration of dial-GTP-modified UMP-kinase with UTP.

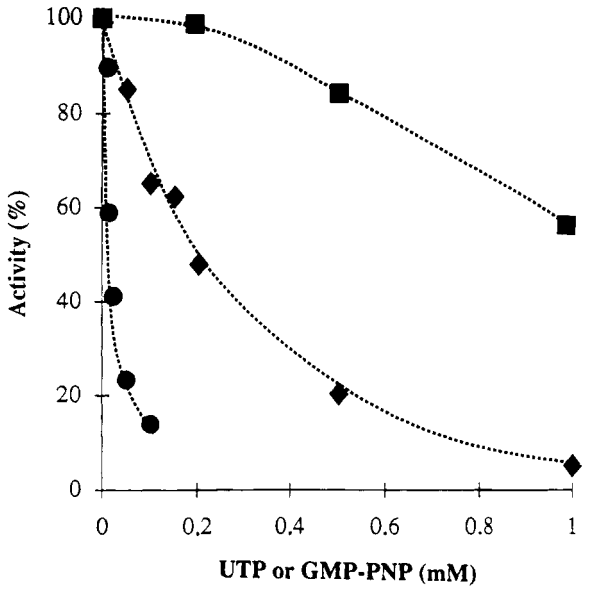

FIGURE 9: Effect of GMP-PNP and UTP on UMP-kinase from *E. coli* in which Asp201 was substituted with Asn. Enzyme activity in the forward reaction was determined with 1 mM ATP + 1 mM UMP and 2 mM $MgCl_2$ as described under Experimental Procedures. Various concentrations of GMP-PNP (■) and UTP (●) were included in the reaction mixture. 100% corresponds to 3.5 units/mg of protein. For comparison, sensitivity of the wild-type enzyme to inhibition by UTP is also represented (♦).

the first 5 min of incubation followed by progressive inactivation (24% of original activity after 90 min of incubation). ATP protected to a significant extent UMP-kinase from inactivation by dial-GTP. When activity at each time of incubation with dial-GTP (normalized as 100%) was compared with that in the presence of GMP-PNP or UTP, respectively, a decrease of the stimulatory/inhibitory effect of the last two nucleotides was observed. These experiments suggest that the binding sites for GTP and UTP might overlap.

Fluorescence Measurements. UMP-kinase from *E. coli*, upon excitation at 295 nm, exhibits a fluorescence emission spectrum with a maximum at 332 nm which indicates that the single tryptophan residue of the protein (Trp119) is located in a hydrophobic environment, not exposed to the solvent. UTP, UDP, and to a lesser extent dUTP enhanced the fluorescence of the protein with no apparent shift of the maximum (Figure 8). The sigmoidal shape of the dose–response curve (Hill number between 3 and 4) indicates strong cooperativity in binding of these three nucleotides to the protein. $MgCl_2$ (between 0.1 and 5 mM) did not affect the fluorescence emission spectrum of UMP-kinase alone, but reversed gradually the enhancing effect of UTP. Thus at 10 μM UTP and 2 mM $MgCl_2$ the fluorescence emission spectrum of UMP-kinase was identical to that of the protein in the absence of nucleotide. A further increase of UTP concentration up to 50 μM was followed again by a 2-fold increase in fluorescence emission, i.e., the maximal value attained with metal-free UTP (data not shown). By varying simultaneously UTP and Mg^{2+} concentrations and by computing the concentrations of Mg^{2+}–UTP and of Mg^{2+}-free UTP as indicated in the caption of Figure 5B, we concluded that only the metal-free UTP was able to increase the fluorescence of Trp119 of bacterial enzyme.

None of the other nucleotides tested (GMP-PNP, ATP, UMP) enhanced the intrinsic fluorescence of UMP-kinase. These nucleotides decreased slightly the fluorescence emission of UMP-kinase. The fluorescence of dial-GTP-modified UMP-kinase was no longer enhanced by UTP.

The effect of UTP on the fluorescence emission of UMP-kinase suggests that, in the absence of this nucleotide, the fluorescence of Trp119 is quenched, probably as a result of the proximity of the indole side chain to a charged amino acid residue. Conformational changes in the protein upon binding of metal-free UTP modify the environment of Trp119, probably by disruption of the interaction with the neighboring charged amino acid residue.

Analysis of an E. coli UMP-kinase Mutant (D201N) with Decreased Activity and Thermal Stability. Among several *smbA* mutants found to be responsible for the altered morphological phenotype of *E. coli* under nonpermissive conditions (Yamanaka et al., 1992), one resulted from substitution of Asp201 by an Asn residue. To find the relationship between the catalytic activity of modified UMP-kinase and altered bacterial phenotype due to this mutation, we overproduced the D201N variant of enzyme in *E. coli* and purified it using the same protocol described for the wild-type enzyme. The specific activity of the D201N mutant in the forward reaction under standard assay conditions, i.e., 1 mM ATP + 1 mM UMP and 2 mM $MgCl_2$ (3.6 units/mg of protein) represented less than 10% of the activity of parent enzyme. The rate of the reverse reaction was 0.13 unit/mg of protein. The apparent K_m values for ATP (0.32 mM) and for UMP (0.11 mM), as well as the Mg^{2+} requirement for optimal activity (6 mM), were higher for the D201N mutant than for the wild-type UMP-kinase. Most significant, however, was the fact that the mutated enzyme was no longer activated by GTP or GMP-PNP (these nucleotides behaved rather like inhibitors), whereas the I_{50} for UTP was 10 times lower than that of the wild-type enzyme (Figure 9). Since

Table 1: Enzymes Involved in the Synthesis of UTP and CTP in *Escherichia coli*

enzyme	gene	oligomeric structure	regulatory nucleotides
carbamoyl phosphate synthetase	*carA, carB*	dimer[a]	UMP, IMP
aspartate carbamoyl transferase	*pyrI, pyrB*	dodecamer[b]	CTP, ATP
dihydrooratase	*pyrC*	dimer	
dihydrooratate oxidase	*pyrD*	dimer	
orotate phosphoribosyl transferase	*pyrE*	dimer	
orotidine phosphate decarboxylase	*pyrF*	dimer	
UMP-kinase	*pyrH*	hexamer	GTP, UTP
CTP-synthetase	*pyrG*	tetramer	GTP, ATP, UTP

[a] Heterodimer. [b] Dodecamer: six catalytic (c) subunits and six regulatory (r) subunits organized as $2c_3$, $3r_2$.

the kinetic properties of the modified UMP-kinase might be explained by structural changes due to Asp → Asn substitution, we examined the thermal stability and the fluorescence properties of the modified enzyme. As shown in Figure 3, the D201N mutant was half-inactivated at 51 °C, whereas the wild-type protein was half-inactivated at 64 °C. Structural changes consecutive to Asp → Asn substitution were also reflected by the fluorescence spectrum of the mutant. The shift of the maximum from 332 to 335 nm was accompanied by an increase (35%) of the quantum yield with respect to the wild-type enzyme. Moreover, UTP, which enhanced by a factor of 2 the fluorescence intensity of the wild-type protein, was ineffective on the D201N mutant. These results indicate that the environment of Trp119 was significantly changed upon substitution of Asp201 with Asn. It is plausible that Asp201 stabilizes the protein through interactions of the carboxylate group and neighboring side chains, including Trp119 itself.

DISCUSSION

The pathways responsible for *de novo* production of nucleotides in various organisms are now well characterized. The genes coding for the enzymes which synthesize UTP and CTP from simple precursors in *E. coli* and various other bacteria, with a single exception, have all been cloned in the last years. Whereas the genetic loci encoding these enzymes are organized as an operon both in *Bacillus subtilis* (Quinn et al., 1991) and *Bacillus caldolyticus*, a thermophilic organism (Ghim et al., 1994), they are unlinked on the chromosome of *E. coli* and *S. typhimurium* (Neuhard & Nygaard, 1987). A common characteristic of pyrimidine nucleotide synthesizing enzymes is their oligomeric structure and, in general, complex control by nucleotides (Table 1). Aspartate carbamoyltransferase from *E. coli* represents, in this respect, the most extensively studied regulatory enzyme (Allewell, 1989; Kantrowicz & Lipscomb, 1990). The missing link in this chain of enzymes was the UMP-kinase, whose gene was identified only recently (Smallshaw & Kelln, 1992). Yamanaka et al. (1992) isolated at the same time the *smbA* gene, whose product was shown to be essential for cell proliferation. The two genes are located at 4 min on the *E. coli* chromosome between the *tsf* and *frr* genes, and the deduced sequences of the two proteins were almost identical. Differences involving six amino acid residues (between Gly63 and Gly70) are most probably attributable to some errors in sequence analysis. Interestingly, the fact that the two gene products were identical escaped the attention of sequence watchers for a year and a half.

The most noteworthy feature of *E. coli* UMP-kinase is the absence of similarity with other nucleoside monophosphate kinases, including UMP-kinase from eukaryotic organisms (Liljelund et al., 1989; Wiesmüller et al., 1990). In fact, the *E. coli* enzyme appears more related to the aspartokinase family, a group of enzymes whose three-dimensional structure has not yet been solved. Yamanaka et al. (1994), although overlooking the link between *pyrH* and *smbA* genes, postulated that the SmbA protein might represent a fourth aspartokinase in *E. coli*. To further substantiate this hypothesis, we built an evolution tree, shown in Figure 10, using the CLUSTAL program (Higgins et al., 1992) (1000 trials for bootstrapping, tried in three independent runs with different random seeds) from the part of the alignment which did not show gaps (no indels). The root was placed along the most divergent branch, corresponding to carbamate kinase from *Pseudomonas aeruginosa*. Nothing is known yet about the residues which are involved in aspartate kinase activity. Obviously, this tree construction will only be validated when appropriate mutations will have identified the conserved residues as involved in regulation or activity of the protein, but we feel confident that most of it will hold, because of the clustering of conserved residues in sequences which all have in common the fact they are kinases. In this tree, three families of enzymes appeared: carbamate kinase, glutamate-5-carboxylate kinase, and aspartokinase. *E. coli* UMP-kinase belongs to the latter family. This observation raises questions related to the evolution and the origin of the first cells. The structure of the tree is better accounted for if one assumes the existence of an ancestral kinase capable of phosphorylating negatively charged substrates possessing either a carboxylate or a phosphate group and having little specificity for the rest of the molecule. This is in line with the hypothesis of Granick (1957), reformulated by Ycas (1974) and more precisely stated by Jensen (1976), that new enzyme functions evolved by recruitment of proteins already catalyzing analogous reactions. The position of UMP-kinase among aspartokinases (aspartate being itself a major building block of pyrimidines nucleotides) would lend further support to an evolution starting with enzymes of poor specificity for substrates. On the other hand, data presented here, and in particular the possible equivalence of carboxylate and phosphate groups as acceptors for an ancestral kinase, supports the model of Granick (1957) and Wächtershäuser (1988) on the origin of metabolism. According to this model, life emerged from a surface metabolism, rather than from a rich but poisonous broth. Appropriate mineral surfaces carrying positive charges can select negatively charged molecules (polycarbonates and phosphates) from an aqueous environment. These molecules can react together, and only those that persist in binding to the surface are kept for further chemical evolution.

The allosteric regulation of UMP-kinase from *E. coli* by nucleotides deserves some additional comments. If the individuality of the substrate binding sites (ATP/ADP and UMP/UDP, respectively) leaves no doubt, the existence of a single site or two separate sites for allosteric effectors remains open to speculation. Some of the experiments presented in this work (fluorescence analysis of wild-type enzyme or kinetic properties of D201N mutant) might argue for binding of UTP and GTP to different sites; allosteric regulation implies conformational adjustments which can affect indirectly the binding ability of each effector by the

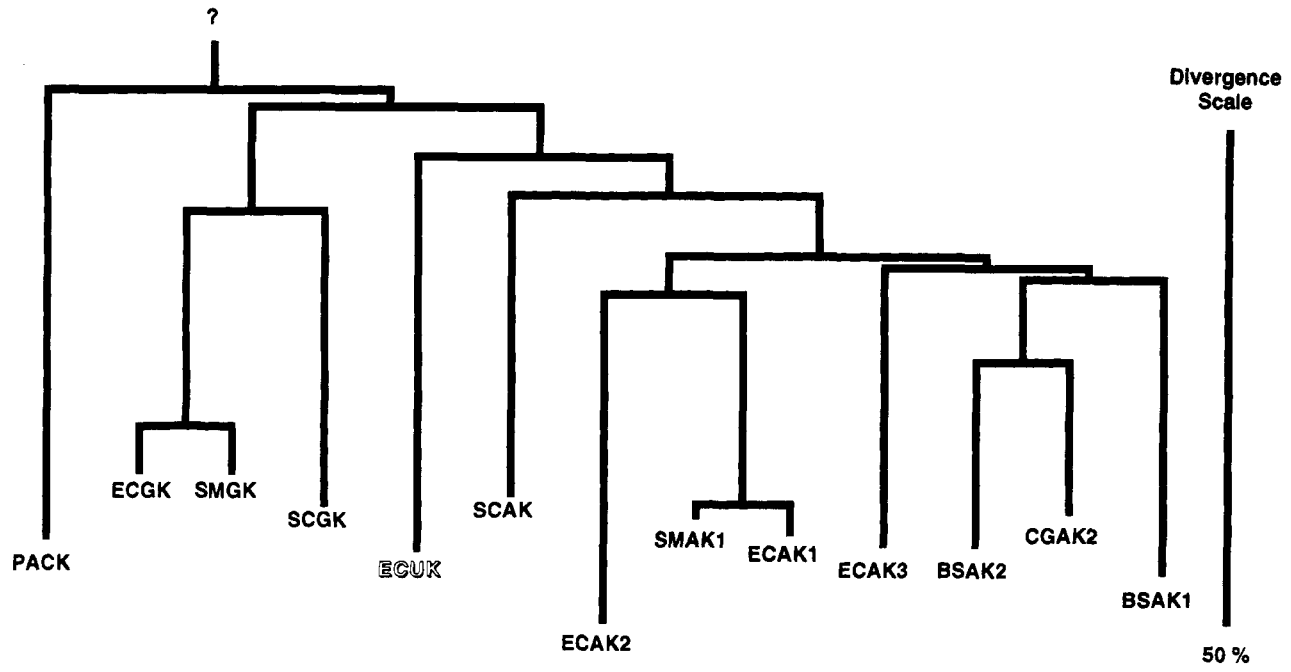

FIGURE 10: Phylogenetic tree of the putative catalytic center of UMP-kinase and other related enzymes (aspartate kinase, glutamate kinase and carbamate kinase). An unrooted phylogenetic tree has been constructed using the CLUSTAL software, with a statistical bootstrap using 1000 randomized sequences. A root (indicated by a question mark) has been proposed on the branch exhibiting the largest divergence score. In this tree UMP-kinase clusters best with the aspartate kinases. PACK, *Pseudomonas aeruginosa* carbamate kinase; ECGK, SMGK, and SCGK, *E. coli*, *S. marcescens* and *S. cerevisiae* glutamate kinase, respectively; ECUK, *E. coli* UMP-kinase; SCAK, *S. cerevisiae* aspartate kinase; ECAK2, BSAK2, and CGAK2, *E. coli*, *B. subtilis*, and *C. glutamicum* aspartate kinase 2, respectively; SMAKI, ECAKI, and BSAKI, *S. marcescens*, *E. coli*, and *B. subtilis* aspartate kinase 1, respectively; ECAK3, *E. coli* aspartate kinase 3.

prior presence of the other. The idea that the GTP and UTP sites overlap (most probably at the level of the sugar and/or phosphate chain) is suggested by the behavior of dial-GTP-modified enzyme. It is worth mentioning in this respect that covalent modification of UMP-kinase after exposure to dial-UTP yields also an "activated" form of enzyme no longer sensitive to GMP-PNP but still inhibited by UTP. Similar results were reported by Boechter and Meister (1981) for carbamoyl phosphate synthetase from *E. coli*, a heterodimer whose activity is regulated by UMP (inhibitor) and IMP (activator). Both dial-UMP and dial-IMP were found to act as potent activators of synthetase.

The opposite effects on conformation and catalysis accompanying the binding of GTP and UTP can result from specific interactions at the level of the heterocycle. A potential site for binding of GTP via the guanine ring is the sequence ^{165}Thr-Lys-Val-Asp^{168}, conserved in GTP-binding proteins as Thr/Asn-Lys-Xaa-Asp, or the G4 region (Bourne et al., 1991). By analogy with Ras proteins, the Asp168 might interact with the exocyclic amino group and the endocyclic N1 of the guanine ring (Pai et al., 1989). A second sequence which might also interact with GTP and is conserved in most GTP-binding proteins as the G3 region (Bourne et al., 1991) is ^{77}Asp-His-Met-Gly^{80}. On the basis of the same analogy with Ras proteins, Asp77 might coordinate the Mg^{2+}, whereas the amide hydrogen of Gly80 can form a hydrogen bond with the γ-phosphate of ATP (Pai et al., 1989).

A last point worthy of comment is the possible involvement of UMP-kinase, i.e., SmbA protein, in cell division. As shown by Yamanaka et al. (1992), expression of the *smbA* gene is essential for cell growth. A point mutation in this gene (*smbA2*) which led to the substitution of Asp201 with Asn induced in *E. coli* a pleiotropic phenotype characterized by cold-sensitive growth, hypersensitivity to SDS, and altered morphology under nonpermissive conditions. Since the *smbA2* mutant encodes an unstable UMP-kinase with impaired catalytic and regulatory functions, we believe that participation of this enzyme in cell proliferation is due mainly to its role in the synthesis of cellular UTP. However, we cannot exclude another function of the *pyrH/smbA* gene product, independent of its catalytic activity. This would not be an unprecedented case, as a human c-myc transcription factor, PuF, was recently identified as a nucleoside diphosphate kinase (Postel et al., 1993) It is therefore of utmost importance to clarify in the near future the mechanism by which UMP-kinase participates directly or indirectly in cell proliferation.

ACKNOWLEDGMENT

We are gratful to N. Glansdorff for the kind gift of *E. coli* strain 14-40-42 used for cloning of the *pyrH* gene, M. Véron for providing us a pure NDP-kinase, J. Bellalou for preparation of various strains of enteric bacteria, G. Batelier for ultracentrifugation analysis, A. Namane for HPLC assay of UMP-kinase activity, G. Cohen and J. Janin for stimulating and helpful discussion, R. A. Kelln for communicating unpublished information, S. Michelson for constructive criticism, and M. Ferrand for excellent secretarial help.

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BI9419341