CMP Kinase from Escherichia coli Is Structurally Related to Other Nucleoside Monophosphate Kinases*

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CMP kinase from Escherichia coli is a monomeric protein of 225 amino acid residues. The protein exhibits little overall sequence similarities with other known NMP kinases. However, residues involved in binding of substrates and/or in catalysis were found conserved, and sequence comparison suggested conservation of the global fold found in adenylate kinases or in several CMP/UMP kinases. The enzyme was purified to homogeneity, crystallized, and analyzed for its structural and catalytic properties. The crystals belong to the hexagonal space group P61, have unit cell parameters a = b = 82.3 Å and c = 60.7 Å, and diffract x-rays to a 1.9 Å resolution. The bacterial enzyme exhibits a fluorescence emission spectrum with maximum at 328 nm upon excitation at 295 nm, which suggests that the single tryptophan residue (Trp35) is located in a hydrophobic environment. Substrate specificity studies showed that CMP kinase from E. coli is active with ATP, dATP, or GTP as donors and with CMP, dCMP, and arabinofuranosyl-CMP as acceptors. This is in contrast with CMP/UMP kinase from Dictyostelium discoideum, an enzyme active on CMP or UMP but much less active on the corresponding deoxynucleotides. Binding of CMP enhanced the affinity of E. coli CMP kinase for ATP or ADP, a particularity never described in this family of proteins that might explain inhibition of enzyme activity by excess of nucleoside monophosphate.

Nucleoside monophosphate (NMP)1 kinases represent an ubiquitous family of catalysts playing a key role in the cell metabolism including synthesis of RNA and DNA molecules (Anderson, 1973; Neuhard and Nygaard, 1987). They catalyze the reversible transfer of phosphoryl group from a NTP (in general ATP) to a NMP. Adenylate kinase represents the best known member of NMP kinase family (Noda, 1973). The adk gene from a great number of living cells was cloned and sequenced, the corresponding protein was purified, and numerous variants obtained by site-directed mutagenesis were characterized for catalytic or structural properties (Tsai and Yan, 1991; Bârzu and Gilles, 1993). Much less is known on the other members of NMP kinase family. Apparently they belong to the adenylate kinase paradigm (Liljelund et al., 1989; Wiesmüller et al., 1990; Konrad, 1992; Müller-Dieckmann and Schulz, 1994) exhibiting sequence similarities and related three-dimensional structure. However, UMP kinase from Escherichia coli and probably from other enteric bacteria deviate from this paradigm. The protein encoded by the pyrH gene (Smailshaw and Kelín, 1992) does not display any sequence similarity to known NMP kinase but belongs to the aspartokinase family (Serina et al., 1995). Moreover, it has an oligomeric structure and is subject to complex regulatory control by GTP and UTP (Serina et al., 1995). Because UMP kinase from E. coli has an absolute specificity for UMP as substrate, the existence in this bacterium of at least one other enzyme acting specifically on CMP was postulated. Fricke et al. (1995) showed that the msa gene from E. coli, whose function was identified as suppressing the conditional lethal phenotype of certain smbA mutants (Yamamaka et al., 1992, 1994), is identical to the cmk gene. The smbA gene itself, shown to be essential for cell proliferation, is identical to the pyrH gene (Smailshaw and Kelín, 1992; Serina et al., 1995). These surprising observations prompted us to undertake a detailed biochemical analysis of CMP kinase from E. coli, purified after overexpression of the corresponding gene.

EXPERIMENTAL PROCEDURES

Chemicals—Adenine, cytidine, and uridine nucleotides, restriction enzymes, T4DNA ligase, and coupling enzymes were from Boehringer Mannheim. T7DNA polymerase and the four nucleoside triphosphates used in sequencing reaction were from Pharmacia. Arabinofuranosyl-CMP was a product of Sigma. Oligonucleotides were synthesized according to the phosphoamidite method using a commercial DNA synthesizer (Cyclone TM Biosearch). Nucleoside-diphosphate kinase (EC 2.7.4.6) from Dictyostelium discoideum (2,000 units/mg of protein) was kindly provided by M. Véron. Ant-dATP, Ant-dADP, Ant-dAMP, and Ant-dCMP were synthesized according to published procedures (Hiratsuka, 1982; Sarfati et al., 1990) from isatoic anhydride and the corresponding deoxynucleoside phosphates.

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g These abbreviations used are: NMP, nucleoside monophosphate; Ant-dATP, Ant-dADP, Ant-dAMP, and Ant-dCMP, 3’-anthraniloyl derivatives of dATP, dADP, dAMP, and dCMP; PAGE, polyacrylamide gel electrophoresis; DTNB, 5,5’-dithiobis(nitrobenzoic acid); TPCK-trypsin, l-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin.
Bacterial Strains, Plasmids, Growth Conditions, and DNA Manipulations—The cmk/mssA gene encoding CMP kinase was amplified by polymerase chain reaction using chromosomal DNA from the E. coli strain NM554 (Raleigh et al., 1988) as the matrix. The polymerase chain reaction product was inserted between the restriction sites of plasmid pET22b (Novagen). The resulting plasmid pHSP210 harbors the promoter/operator region, consisting of T7 promoter followed by a operator. The sequence of the cmk gene was verified by the dideoxynucleotide sequencing method (Sanger et al., 1977). Introducing plasmid pHSP210 into E. coli strain BL21 (DE3) (Novagen) that produces T7 RNA polymerase enables the synthesis of high amounts (approximately 30% of total E. coli proteins). Strain BL21 (DE3) pHSP210 was grown at 37°C in 2YT medium (Sambrook et al., 1989) containing 150 μg/ml ampicillin until A600 reached 1.5. Then isopropyl-1-thio-β-D-galactoside (final concentration, 1 mM) was added, and the culture was further incubated at 37°C for 3 h.

Purification of CMP Kinase and Activity Assays—Enzyme from the CMP kinase overproducing strain was purified by a two-step procedure involving chromatography on blue Sepharose and Ultrogel AcA54 (Bärzu and Michelson, 1983) with the following modification: the enzyme retained on the blue Sepharose was eluted with 50 mM Tris-HCl (pH 7.4) containing 1M NaCl. CMP kinase activity was determined in a simple quadrupole mass spectrometer API-I (Perkin-Elmer, Toronto, Canada) equipped with an ion spray (nebulizer-assisted electrospray) source. The sample (~20 pmol/μl) dissolved in 20% acetonitrile in water and 0.1% HCOOH, was delivered to the source at a flow rate of 5 μl/min utilizing a medical infusion pump (Model 11, Harvard Apparatus, South Natick, MA). Polypropylene glycol was used to calibrate the quadrupole. Ion spray mass spectra were acquired by scanning from m/z 600 to m/z 2000 with a step size of 0.1 dalton and a dwell time of 10 ms. The potential of the spray needle was held at 5.0 kV, and the spectra were recorded at an orifice voltage of 60 V. Mac Bio spec was the computer program for calculation of the molecular mass of CMP kinase.

Fluorescence Measurements—The emission spectrum of CMP kinase (λem = 295 nm; bandwidth, 5 nm) was recorded from 305 to 400 nm using a Perkin-Elmer LS-58 luminescence spectrometer thermostated at 25°C using a 1 x 1-cm UV-grade quartz cuvette (sample volume, 2 ml). Binding of nucleotides to E. coli enzyme was measured from the fluorescence of Ant-dATP or of Ant-dADP (λexc = 330 nm; λem = 420 nm) (Sarfati et al., 1990).

Other Analytical Procedures—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-PAGE was performed as described by Laemmli (1970). Protein bands from SDS-PAGE were electrophotographed onto a Problott membrane filter (Applied Biosystems) and detected by staining in the Coomasie Blue. The N-terminal amino acid sequence of the protein from the excised band was determined by a protein sequencer (Applied Biosystems Inc.).
RESULTS

Sequence Comparison of E. coli CMP Kinase with Other Members of NMP Kinase Family—CMP kinase from E. coli and from Bacillus subtilis and Mycobacterium leprae are closely related but showed little overall sequence similarities with other NMP kinases with known three-dimensional structures (Fig. 1). However, sequence alignment points out the conservation of residues involved in binding of substrates or in catalysis, as well as conservative replacements in several long stretches of amino acids, suggesting conservation of the same global fold in all these proteins. The characteristic connectivities (-2x, +1x, +2x, +1x), according to the nomenclature of Richardson (1976), linking the five β-strands are predicted to be conserved in bacterial CMP kinase. The numbering of the secondary structure refers to that of the related Rossmann fold (Rossmann et al., 1974). An insertion of 32–36 amino acids between helices α2' and α3 was present in CMP kinase from E. coli, B. subtilis, and M. leprae. The NMP kinase signature comprises few conserved regions recognized as involved in interaction with phosphate groups, nucleotide bases, and Mg2+ ions. The consensus sequence comprising the β1-strand and the phosphate binding loop (P loop) is X_hX_hX_hGXXgX_gKgt, where X stands for any amino acid and X_h stands for any hydrophobic amino acid residue. The next conserved segment (β2-α3 loop) has the consensus sequence X_hX_hXX_hX_gG, and a third stretch of similarity can be ascribed as the Mg2+-binding loop: X_hX_hX_hG.

From these structural comparisons one might predict that the active site in the bacterial CMP kinase would be similar to that described for different adenylate kinases or for UMP kinases from yeast or D. discoideum.

Overexpression and Molecular Characterization of CMP Kinase from E. coli—The CMP kinase from E. coli was overproduced in strain BL21 (DE3). The protein was purified by blue Sepharose and Ultrogel AcA54 chromatography (Fig. 2). The first four N-terminal amino acid residues (Thr-Ala-Ile-Ala) corresponded to those deduced from the cmk/mssA gene, except that the N-terminal methionine residue was missing. The molecular mass of the protein determined by electrospray ionization mass spectrometry (Fig. 3) was 24,617, which is 31 units higher than that deduced from the published nucleotide sequence (24,586 daltons). The nucleotide sequence of the cmk gene amplified by polymerase chain reaction revealed that codon 164 differed from the published sequence of the cmk/mssA gene (Yamanaka et al., 1994; EMBL accession X00785). Two independent plasmids harbored the same Val164 → Glu mutation, thus suggesting either a very early polymerase chain reaction-induced mutation event prior to the cloning step or a genuine difference between the cmk genes from strains NM554 and W3110. Fourteen independent sequence determinations were performed on our plasmids. The replacement Val164 → Glu accounted for the differences in the deduced molecular mass of wild-type protein and that determined experimentally on the protein overproduced in E. coli. Substitution of Val164 by a Glu residue apparently does not affect the catalytic properties of E. coli CMP kinase, which is not surprising. Val164 is not conserved in any other NMP kinase; it corresponds to a Lys residue in B. subtilis enzyme and to Ala in M. leprae CMP kinase.

CMP kinase from E. coli has a single Cys residue (Cys21) that is conserved in adenylate kinase 1 and UMP kinase from D. discoideum but not in CMP kinase from B. subtilis and M. leprae. This residue reacted with DTNB under native conditions at low rates (0.2 SH/mol enzyme at pH 7.4 and 10 min of incubation at room temperature). In the presence of SDS, the protein reacted rapidly with DTNB, the thionitrobenzoate/protein ratio being ≥ 0.9. It seems therefore that the single thiol of CMP kinase is less exposed to the solvent. The same is true for the single Trp residue (Trp99, according to the numbering in protein sequence). Upon excitation at 295 nm, E. coli CMP
kinase exhibits a fluorescence emission spectrum with maximum at 328 nm. Guanidinium hydrochloride at concentrations higher than 0.6 mM shifted the fluorescence maximum to higher wavelengths with no decrease of the maximum amplitude. The midpoint transition for CMP kinase from E. coli is at 0.9 M of guanidinium hydrochloride (Fig. 4).

Thermal denaturation experiments indicated that CMP kinase was half-inactivated at 52°C (not shown). The first order rate constant (3.1 x 10^-3 s^-1) of inactivation of CMP kinase by TPCK-trypsin was in good agreement with the decrease in absorption of the enzyme band scanned after SDS-PAGE and Coomassie Blue staining. ATP (as well as ADP, CDP, CTP, and to a lesser extent CMP) exerted significant protection against protolysis (Fig. 5). The N-terminal sequencing of various peptides after electrophoretic transfer onto nitrocellulose membrane filter suggested that TPCK-trypsin cleaves CMP kinase from its C-terminal end, rich in lysine and arginine residues. The fragment marked with an asterisk in Fig. 5 that is resistant to further proteolytic cleavage corresponds most probably to a C-terminal truncated form of CMP kinase ending with amino acids AHRR.

Crystallization and Preliminary X-ray Diffraction Studies of CMP Kinase—The protein crystallized in ammonium sulfate. Large hexagonal-shaped colorless crystals as shown in Fig. 6 appeared at 20°C at either pH 6.5 or 7.4. They grew over a period of 2 weeks. The largest crystal was obtained with a drop (6 μl) containing 23 mg protein/ml and 0.4 M ammonium sulfate in 50 mM Tris-HCl buffer (pH 7.4) over a pit (1 ml) containing 1.2 M ammonium sulfate. Its size was 0.4 mm. It was sealed in a glass capillary with 1.4 M ammonium sulfate.

The unit cell parameters are a = b = 82.3 Å, c = 60.7 Å, α = β = 90°, and γ = 120° (V = 356,000 Å³). Assuming that the unit cell contains six molecules (one per asymmetric unit), the Vₚ value is calculated as 2.4 Å³ Da⁻¹, resulting in a solvent content of 49%. These values are typical of protein crystals (Matthews, 1968). Intensity data were scaled and merged up to 1.9 Å resolution. Consequently, a total of 17744 independent reflections was obtained, which corresponds to 95.6% of the number of theoretically possible reflections. The merging R factor is 0.052 for 95,706 measurements (Rmerge = Σh|I_h| - Σh|I(h)| / Σh|I(h)|, where |I(h)| is the mean intensity of a reflection (h) and I(h) is the jth measurement of reflection h).

Catalytic Properties of CMP Kinase from E. coli—The reaction rates of CMP kinase from E. coli with various nucleotides as substrates indicated that ATP, dATP, and GTP are good phosphoryl donors, whereas ITP is a poor substrate (Table I). The activity with other nucleoside triphosphates (CTP, dCTP, UTP, or dUTP) is still measurable but less than 0.05% of that with ATP. From the NMPs tested CMP, dCMP, and arabinofuranosyl-CMP are by far the best phosphate acceptors (Table II). Whereas UMP, 2-thiouridine 5’-monophosphate, and dUMP can still act as poor substrates, TMP, Ant-dCMP, 5-methyl-CMP, AMP, dAMP, GMP, and dGMP were neither substrates nor inhibitors of the bacterial enzyme.
exerted a slight inhibitory effect over 0.3 mM (calculated values, \(K_i\) values, 3.0 M). The concentration of ATP was kept constant at 1 mM, whereas the concentration of nucleoside monophosphates was varied within 0.025 and 2 mM.

TABLE II

| Nucleoside monophosphate | \(V_m\) | \(K_m\) |
|--------------------------|--------|--------|
| ATP                      | 189    | 0.038  |
| dATP                     | 175    | 0.087  |
| GTP                      | 233    | 0.64   |
| ITP                      | 6.2    | 0.87   |

The reaction rates with CMP and dCMP were best fitted with the equation:

\[
v = V_m \cdot S / (K_m + S + S^2/K_i).
\]

Emission spectra of 3'-anthraniloyl derivatives of dATP, dADP, dAMP, or dCMP in 50 mM Tris-HCl (pH 7.4) showed a maximum at 425 nm upon excitation at 330 nm. The addition of CMP kinase in a 10-fold excess to aqueous solutions of fluorescent nucleotides increased the fluorescence intensity of Ant-dATP and Ant-dADP by a factor higher than 3 (Fig. 8). The same concentration of protein enhanced the fluorescence of Ant-dAMP by only 25%, the effect being even less (11% increase in fluorescence intensity) with Ant-dCMP. Determination of fluorescence intensities of Ant-dATP or Ant-dADP with various concentrations of CMP kinase allowed calculation of \(K_d\) values for Ant-dATP/CMP kinase or Ant-dADP/CMP kinase complexes. Specificity of binding of these nucleotides to CMP kinase was confirmed by the fact that excess of ATP or ADP completely displaced the fluorescent nucleotides from the active site of CMP kinase. From each individual point of the "titration" curve with natural nucleotides, the \(K_d\) values for ATP or ADP were calculated (Fig. 8 and Table III). The most striking observation from these experiments was that CMP (as well as dCMP) significantly enhanced the affinity of CMP kinase for the corresponding co-substrates. These fluorescence experiments are consistent with the kinetic studies and suggest that CMP inhibition is caused by an abortive complex that prevents the release of product (Bell and Bell, 1988).

**DISCUSSION**

De novo synthesis and recycling of nucleotides in bacteria and eukaryotes are quite well understood processes. It is generally assumed that phosphorylation to nucleoside diphos-
phates is a specific reaction and that NMP kinases represent a homogeneous family of catalysts sharing similar primary and three-dimensional structure with adenylate kinases. It was therefore a surprise to discover that UMP kinase in E. coli is an enzyme of completely different descent being related to aspar-токинases rather than to adenylate kinases (Serina et al., 1995).

In addition to the highly specific UMP kinase, enteric bacteria contain two other pyrimidine NMP kinases: a TMP kinase and a CMP kinase. Mutants defective in the two enzyme activities were isolated and characterized either in E. coli or in S. typhi-murium (Beck et al., 1974; Blinkley and Kuenpm, 1986). However, only recently Fricker et al. (1995), corroborating previous works on an E. coli gene specifying a 25-kDa polypeptide (Pedersen et al., 1984) and recent works on a mssA gene (Yamanaka et al., 1994) (from multicopy suppressor of smba), demonstrated that cmk and mssA are identical genes. The known E. coli cmk gene is not essential (contrary to the adk or pyrH genes), but this may be due to the presence of a second cmk gene, as found in Haemophilus influenzae, a close relative of E. coli (Fleischmann et al., 1995). Cytidine nucleotides have a special situation in the nucleotide metabolism because CTP results in the de novo pathway from UTP and not from the corresponding monophosphate and diphosphate precursors. This is particularly important because deoxyribozymes result by reduction of the corresponding ribonucleoside diphosphates. Scavenging of CMP and production of CDp are therefore steps of major importance for DNA synthesis. This accounts for the observation that the DNA replication rate is reduced in cmk mutants where CMP and dCMP accumulate at high levels. The fact that in high gene copy number the cmk/mssA gene can suppress defects in the smba/pyrH gene (Yamanaka et al., 1994) implies that E. coli CMP kinase is endowed with a residual UMP kinase activity, which indeed was the case as shown in this paper.

Although CMP is not produced in the de novo pathway, it might accumulate either from CTP during the synthesis of phospholipids or from the hydrolytic cleavage of mRNA. Therefore, the physiological role of CMP kinase is to recycle CMP to CDp, which is either rapidly phosphorylated by the unspecific nucleoside-diphosphate kinase to CTP or reduced to dCDp. In bacteria CDp (as well as ADP, UDP, or GDP) can also result from phosphorolytic cleavage of mRNA by polynucleotide phosphorylase (EC 2.7.7.8) (Carpousis et al., 1994; Py et al., 1994). It therefore seems worth looking for a possible link between these two CDp-producing enzymes, i.e. CMP kinase and polynucleotide phosphorylase. The cmk gene is located in the E. coli chromosome as the first gene of an operon comprising the rpsA gene (Fricke et al., 1995). In this organism, the rpsA gene product (protein S1) promotes translation initiation by binding to the 5' end of the mRNA molecule and enhancing the recognition of the ribosome binding site upstream of the start codon. Consulting of the data base present at the Institute for Genomic Research site (http://www.tigr.org), we found that the same organization holds for one of the cmk genes present in H. influenzae (Fleischmann et al., 1995). Surprisingly, the same is also true for B. subtillis, a Gram-positive organism, described as not possessing a ribosomal S1 protein. Comparison of S1 sequence with data libraries revealed that it possessed an internal repetition motif of 69 residues that is also present in polynucleotide phosphorylase (Regnier et al., 1987). In addition, further analysis demonstrates that this motif is also present in a RNA helicase molecule. This permits us to propose that the primary function of S1 is to present RNA molecules to polynucleotide phosphorylase, so that they can be degraded efficiently from their 3' end. This is consistent with the newly discovered complex of RNA degradation, comprising polynucleotide phosphorylase (Carpousis et al., 1994). In E. coli, this function has evolved, as a side effect, to that of presenting the mRNA to the ribosome, under a conformation adapted to translation initiation. The selection pressure linked to this function has associated S1 to the cmk gene product, because this ends in the same general function, generation of CDP (Company et al., 1991).

Sequence comparison of E. coli CMP kinase with other members of the NMP kinase family showed few overall sequence similarities. However, the protein seems to conserve the same global fold as found in the NMP kinases whose three-dimensional structure was already solved (Muller-Dieckmann and Schulz, 1995; Vonhein et al., 1995). Molecular modelling of E. coli CMP kinase showed that Cys23 and Trp30 are buried in the protein core at the interface of the helix a1 and the beta-strand, in agreement with experiments of intrinsic fluorescence of the protein or reactivity toward DTNB. These two amino acid residues are conserved in UMP/CMP kinase from D. discoideum as Cys23 and Trp32. Because the latter enzyme has a second Cys residue at the position 119 and was readily inactivated by DTNB (Wiesmuller et al., 1990), we might deduce that the DTNB-sensitive thiol group in the D. discoideum enzyme corresponds to Cys119. In the same way we can deduce that Arg158 in E. coli CMP kinase which is exposed to the solvent in nucleotide-free form of protein is stacked against the substrate base rings in the nucleotide-complexed CMP kinase, explaining the protection of enzyme against trypsin digestion by ATP or ADP.

Another residue found conserved as threonine in adenylate kinases (Thr39 in pig muscle adenylyl kinase 1 and Thr31 in E. coli enzyme) and as alanine in yeast (Ala47), D. discoideum (Ala37), or porcine brain (Ala34) UMP/CMP kinase (Okajima et al., 1995) deserves some comments. These residues were suggested by several authors (Muller-Dieckmann and Schulz, 1995; Okajima et al., 1993) to play a role in recognition of the heterocycle and therefore to contribute to the substrate specificity of NMP kinases. Contrary to expectations, in the E. coli, B. subtillis, and M. leprae CMP kinase the same position is occupied by Ser/Thr residues, which are characteristic to the adenylate kinase family. In fact, site-directed mutagenesis of Ala27 to Thr in D. discoideum UMP/CMP kinase did not change the substrate specificity of the slime mold enzyme. The determination of the three-dimensional structure of the CMP kinase from E. coli is expected to answer more precisely all these questions and also to explain differences in substrate specificity as compared with the enzymes from yeast or from D. discoideum (Wiesmüller et al., 1995).

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