Title: Phosphorylation of Cytidine-, Deoxycytidine-, and Their Analog-Monophosphates by Human UMP/CMP Kinase Is Differentially Regulated by ATP and Magnesium

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Running Title: Phosphorylation of dCMP and analog monophosphates regulated by ATP and Mg

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Abbreviations: AMP, adenosine monophosphate; ATP, adenosine triphosphate; ara-C, 1-β-D-arabinofuranosylcytosine; CMP, cytidine monophosphate; dCMP, deoxycytidine monophosphate; ddC, β-D-2’, 3’-dideoxyctydine; dUMP, deoxyuridine monophosphate; DTT, dithiothreitol; FdUMP, 5-fluorodeoxyuridine monophosphate; gemcitabine or dFdC, 2’, 2’-difluorodeoxycytidine; HPLC, high performance liquid chromatography; L-ddC, L-2’, 3’-dideoxyctydine; L-OddC, β-L-dioxaolanecytidine; L-SSdC or 3-TC , β-L-2’, 3’-dideoxy-3’-thiacytidine; Mg, magnesium; MP, monophosphate; NMP, nucleoside monophosphate; UMP, uridine monophosphate.
ABSTRACT

Human UMP/CMP kinase (EC 2.7.4.14) is responsible for phosphorylation of CMP, UMP, and dCMP and also plays an important role in the activation of pyrimidine analogs, some of which are clinically useful anticancer or antiviral drugs. Previous kinetic data using recombinant or highly purified human UMP/CMP kinase showed that dCMP, as well as pyrimidine analog monophosphates, were much poorer substrates than CMP or UMP for this enzyme. This implies that other unidentified mechanisms must be involved to make phosphorylation of dCMP or pyrimidine analog monophosphates inside cells by this enzyme possible. Here, we re-evaluated the optimal reaction conditions for human recombinant human UMP/CMP kinase to phosphorylate dCMP and CMP (referred as dCMPK and CMPK activities). We found that ATP and magnesium (Mg) were important regulators of the kinase activities of this enzyme. Free Mg enhanced dCMPK activity but inhibited CMPK activity. Free ATP or excess ATP/Mg, on the other hand, inhibited dCMPK but not CMPK reactions. The differential regulation of dCMPK versus CMPK activities by ATP or Mg was also seen in other 2'-deoxy-pyrimidine analog monophosphates (dUMP, 5FdUMP, ara-C monophosphate, gemcitabine monophosphate) versus their
ribose-counterparts (UMP, 5FUMP), in a similar manner. The data suggest that the active sites of human UMP/CMP kinase for dCMP and for CMP cannot be identical. Furthermore, enzyme inhibition studies demonstrated that CMP could inhibit dCMP phosphorylation in a non-competitive manner, with $K_i$ values much higher than its own $K_m$ values. We thus propose novel models for the phosphorylation action of human UMP/CMP kinase.
INTRODUCTION

UMP/CMP kinase (EC 2.7.4.14), which phosphorylates CMP, UMP, and dCMP to their respective diphosphates, is crucial for cellular nucleic acid synthesis. The synthesis of pyrimidine nucleotides, in both de novo and salvage pathways, requires this enzyme to produce diphosphates from the monophosphate forms (Van Rompay et al, 2000). Recently, a conditional lethal mutant isolated from Saccharomyces cerevisiae was identified to be caused by mutated UMP/CMP kinase, indicating the essentiality of this enzyme in the survival of this organism and possibly in mammalian cells (Liljelund and Lacroute, 1986).

UMP/CMP kinase also plays an important role in the activation of deoxycytidine analogs, many of which are important anti-cancer and anti-viral agents (Cheng, 2001; Galmarini et al, 2001 & 2002). For example, 1-β-D-arabinofuranosylcytosine (ara-C) is commonly used to treat hematological malignancies (Grant, 1998). 2’, 2’-difluorodeoxycytidine (gemcitabine) has been shown to be active against pancreatic cancer and several other solid tumors (Hui and Reitz, 1997; Noble and Goa, 1997). β-L-dioxolanecytidine (L-OddC), a deoxycytidine analog with an unnatural L-configuration, is currently under active clinical investigations because of
promising anti-tumor effects seen in preclinical models and early clinical studies (Grove et al, 1995; Grove and Cheng, 1996; Weitman et al, 2000; Townsley et al, 2003). Further, β-D-2′, 3′-dideoxycytidine (ddC) and β-L-2′, 3′-dideoxy-3′-thiacytidine (L-SSdC, 3-TC, or lamivudine) are active anti-human immunodeficiency virus (HIV) and anti-human hepatitis B virus (HBV) agents (Cheng, 2001). The analogs need to be phosphorylated stepwise to their triphosphate forms to exert their therapeutic effects. UMP/CMP kinase is responsible for the phosphorylation of these analogs from monophosphates to their diphosphate metabolites (Liou et al, 2002).

Recently, human UMP/CMP kinase has been cloned and characterized by several groups, including ours (Van Rompay et al, 1999; Pearman et al, 2001; Liou et al, 2002; Pasti et al, 2003). Kinetic studies of recombinant human UMP/CMP kinase have shown that dCMP is a much poorer substrate than CMP or UMP (Van Rompay et al, 1999; Liou et al, 2002; Pasti et al, 2003). The relative efficiency of phosphorylating dCMP by this enzyme is about 100-fold less efficient than that of CMP or UMP. This finding is consistent with previous studies using partially purified enzymes from human cancer cells (Teng et al, 1976; Hande and Chabner, 1978; Scott and Wright, 1979). The phosphorylation of various deoxycytidine analog monophosphates could be
carried out by recombinant human UMP/CMP kinase in vitro, but only with efficiencies comparable to or less than the phosphorylation rate of dCMP (Liou et al, 2002). Given the fact that the intracellular UMP or CMP concentration is much higher than dCMP (Traut, 1994a), the kinetic properties of recombinant human UMP/CMP kinase in vitro raises a question of how dCMP can be phosphorylated by this enzyme inside cells. It also implies that other cellular mechanisms which potentially lead to an improved phosphorylation rate of dCMP and other deoxycytidine analog monophosphates do exist inside cells. Several hypotheses, such as post-translational modifications of the protein, interactions with other cellular proteins, and the presence of un-identified cellular dCMP kinases, have yet to be proven (Liou et al, 2002).

In this report, we started with a careful re-evaluation of the kinetic properties of human recombinant UMP/CMP kinase and found out that the phosphorylation of CMP or dCMP by this enzyme was regulated by ATP and magnesium (Mg) very differently. We went on to demonstrate that different concentrations of ATP and Mg could change the kinetic parameters of this enzyme. The enzyme inhibition studies showed that CMP inhibited the phosphorylation of dCMP by this enzyme in a non-competitive manner, with $K_i$ values much higher than its own $K_m$ values. Our data indicate that the
binding sites for CMP or dCMP of human UMP/CMP kinase cannot be identical.
MATERIALS AND METHODS

Nucleoside and Nucleoside Analog Monophosphates. CMP, dCMP, UMP, dUMP, 5-fluorodeoxyuridine monophosphate (FdUMP), ara-CMP, and ATP were purchased from Sigma-Aldrich Corp. (St. Louis, MO). 5-Fluorouridine monophosphate (FUMP), [5-3H]-CMP, [5-3H]-dCMP, [5-3H]-UMP, and [5-3H]-dUMP were purchased from Moravek Biochemicals Inc. (Brea, CA).

The monophosphates of other nucleoside analog, including gemcitabine, D-2', 3'-dideoxycytidine (ddC), L-2', 3'-dideoxycytidine (L-ddC), L-SddC, and L-OddC, were synthesized and purified according to the procedures published before with minor modifications (Ruth and Cheng, 1981).

Recombinant human UMP/CMP kinase. Human UMP/CMP kinase was cloned from KB cells, a human oropharyngeal carcinoma cell line (Liou et al, 2002). Based on pET-28a expression vector (Novagen Inc., Madison, WI), the protein was expressed with an N-terminal His-tag/thrombin configuration. The details of expression, purification, and thrombin cutting were previously reported (Liou et al, 2002). In short, BL21-Gold (DE3)-competent E. coli cells (Stratagene Corp., La Jolla, CA) were transformed with pET-28a-human UMP/CMP kinase construct. After treatment with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for the induction of protein expression,
the transformants were disrupted in a lysis buffer [40 mM Tris-HCl (pH 7.5), 10 mM NaCl, 5 mM NaF, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride] by sonication. The recombinant protein was purified by Ni\(^{2+}\)-column chromatography (Invitrogen Corp., Carlsbad, CA). The His-tag of recombinant protein was cut out by the treatment of biotinylated thrombin (Invitrogen), which was later removed by avidin-agarose beads according to the manufacturer’s instructions (Invitrogen). After an additional pass-through of Ni\(^{2+}\)-column to remove the free His-tag digested out by thrombin treatment, the purified proteins were obtained with greater than 95% of purity as determined by silver staining.

**Enzyme Activity Assays.** Two methods were used to determine enzyme activities: the DE-81 disc (Whatman Inc., Clifton, NJ) assay and a HPLC assay. The DE81 disc assay was performed according to previous reports (Cheng and Prusoff, 1974; Liou et al, 2002), when \(^3\)H-labeled monophosphate materials were available. The enzyme assays was performed under different concentrations of substrates, ATP, or magnesium in a buffer consisting of 50 mM Tri-HCl pH 7.5, 10 mM NaF, 2 mM DTT, supplemented with creatine phosphate and creatine kinase for regeneration of ATP in a total volume of 75 \(\mu\)l for each reaction. The reaction was performed at 37°C for 20 to 120
minutes (min), and was stopped by being chilled in ice. A 50-µl aliquot from each reaction mixture was spotted onto DE-81 discs (Whatman). The discs were washed 3 times with washing solutions (1 mM ammonium formate plus 50 mM formic acid for CMP and dCMP; 1 mM ammonium formate plus 0.5 M formic acid for UMP and dUMP) for 3 min, once with 95% ethanol for 3 min, and subsequently dried. In order to improve the detection of [³H]-labeled radioactive nucleotides, compounds were eluted from the discs by incubation with one ml 0.1N HCl containing 2 M NaCl for 20 min before reading by a scintillation counter (Beckman Coulter Inc., Fullerton, CA). The enzyme activities were defined as nmol/min/mg protein.

For other analog monophosphates, the reactions were performed in the above-mentioned buffers without ATP-regenerating system. Reactions were terminated by addition of a half-volume of 45% trichloroacetic acid. After extraction by half-volume of trioctylamine-trichlorotrifluoroethane (45:55, v/v) twice, samples were then analyzed by HPLC (Shimadzu America Inc., Columbia, MD) in a binary gradient of water and potassium phosphate buffer using an anion exchange column (Partisil-SAX, Whatman) (Krishnan et al, 2002).
RESULTS

ATP and Mg regulate the CMPK or dCMPK activities of recombinant human UMP/CMP kinase very differently. The effects of ATP, ATP/Mg, and Mg on phosphorylation of CMP or dCMP by recombinant protein were systematically evaluated. As shown in Fig.1-A, the phosphorylation rate of CMP by recombinant protein (referred as CMPK activity) increased with the concentration of ATP and achieved an optimum at 2 mM of ATP, in the presence of 2 mM Mg. CMPK activity did not decrease until the ATP concentration was increased to more than 5 mM. On the other hand, although the phosphorylation of dCMP by recombinant protein (referred as dCMPK activity) also increased when ATP was increased from 0 to 1 mM, further an increase of free ATP had a markedly inhibitory effect. When ATP was more than 5 mM (free ATP was more than 3 mM), dCMPK activity decreased to less than 10% of its optimum activity.

In terms of the impact of ATP/Mg (in equal concentrations) on CMPK and dCMPK activities of recombinant human UMP/CMP kinase, while both CMPK and dCMPK activities achieved maximal when ATP/Mg was 1 ~ 2 mM, further increases of ATP/Mg significantly suppressed dCMPK activity, but had no inhibition on CMPK activity (Fig. 1-B).
The requirement for Mg in the kinase activity was much more stringent in
dCMPK reaction than in CMPK reaction. Under 0 mM of Mg, CMPK and
dCMPK activities were ~70 and 0 µmol/min/mg protein, respectively. The
optimum of CMPK reaction (in the presence of 2 mM ATP) was achieved when
Mg was 2 mM, and further increases of Mg concentration resulted in a mild
dose-dependent inhibition (Fig. 1-C). In contrast, the optimum of dCMPK
reaction was at 4 to 6 mM of Mg, i.e. there was 2~4 mM of free Mg in reactions.
Further increase of free Mg might have some inhibitory effect on dCMPK
activity (Fig. 1-C).

Change of ATP/Mg leads to a change of kinetic properties of human
recombinant UMP/CMP kinase. Our preliminary observations showed that
free ATP and excessive amount of ATP/Mg have a significant inhibition on
dCMPK activity but not on CMPK activity of recombinant human UMP/CMP
kinase, and free Mg has enhancing and suppressing effects on dCMPK activity
and CMPK activity, respectively. We wondered whether an increase of free
Mg and a decrease of free ATP would change the kinetics of UMP/CMP kinase
and make it more favorable to the phosphorylation of dCMP. The $K_m$ and
relative activity in terms of phosphorylating dCMP and CMP were evaluated
under different concentrations of ATP/Mg, shown in Table 1.
When ATP/Mg concentrations were changed from 8/8 mM, which was quite unfavorable to dCMP phosphorylation, to 0.1/2 mM or 0.5/2 mM, which was relatively more favorable to dCMP phosphorylation, the $K_m$s for dCMP decreased and the relative activity increased. As a result, the relative efficiency for this enzyme to phosphorylate dCMP increased 4.9-fold. On the other hand, the relative efficiency of recombinant protein to phosphorylate CMP was also increased for 4.9-fold by changing ATP/Mg from 8/8 mM to 0.1/2 mM (Table 1). In other words, the enzyme kinetic studies demonstrated that the change of ATP/Mg had a comparable and parallel impact on CMPK and dCMPK activities (Table 1). Therefore, this improved kinetic property for dCMP phosphorylation by changing ATP and Mg concentrations could not be the explanation for what we have observed on the different regulatory effects of ATP and Mg in phosphorylating dCMP or CMP.

**Enzyme inhibition studies show that CMP non-competitively inhibited dCMP phosphorylation by recombinant human UMP/CMP kinase.** The different regulation patterns by ATP and Mg in phosphorylating dCMP and CMP raised the question whether human UMP/CMP kinase has a single and identical binding site for both substrates. To answer this question, we used enzyme inhibition studies to understand the characteristics of the active site of
this enzyme. First, we tested CMP as an inhibitor for UMP phosphorylation reaction of recombinant human UMP/CMP kinase. As shown in Fig. 2, CMP inhibited UMP kinase reactions with a competitive-inhibition pattern. The $K_i$ values for CMP to inhibit UMPK reaction were comparable to $K_m$ values of CMP (Table 2). The results indicate that CMP and UMP compete with each other at the same active site of the enzyme. However, when CMP was tested as an inhibitor for dCMP phosphorylation of recombinant protein, CMP was showed to be a non-competitive inhibitor of dCMP reaction (Fig 3). The $K_i$ values for CMP to inhibit dCMP phosphorylation were 4 to 6-fold higher than its own $K_m$ values (Table 2). UMP was also a noncompetitive inhibitor of dCMP reaction (data not shown). The data suggests that CMP or UMP inhibits recombinant protein from phosphorylating dCMP through sites other than the site responsible for its own binding site. Interestingly, dCMP was found to be a competitive inhibitor of CMP phosphorylation (Fig. 4), and the $K_i$ values for dCMP to inhibit CMPK reaction were similar to its own $K_m$ values (Table 3).

In summary, the enzyme inhibition studies showed that the active sites for dCMP and CMP of human recombinant UMP/CMP kinase cannot be identical.

**Phosphorylation of pyrimidine nucleoside analog monophosphates was**
also regulated by ATP and Mg. Since many pyrimidine nucleoside analog monophosphates can be phosphorylated by human UMP/CMP kinase, we evaluated whether the phosphorylation of these analogs could be regulated by ATP and Mg in a similar fashion as the naturally occurring pyrimidine monophosphates. Using an approach shown in Fig. 5, we evaluated the modulatory effects of free ATP, free Mg, and ATP/Mg by comparing the differences of phosphorylation rates from reaction conditions with ATP/Mg 5/2 mM versus 2/2 mM, 0.5/2 mM versus 0.5/0.5 mM, and 5/5 mM versus 2/2 mM, respectively. The results are in Table 4. It is of interest to note that the patterns of regulation by ATP and Mg were generally related to the 2'-position of ribose. The phosphorylation of dUMP and FdUMP was enhanced by free Mg, and suppressed by free ATP or excessive amounts of ATP/Mg, in a pattern similar to dCMP phosphorylation (Fig. 5). On the other hand, phosphorylation of UMP or FUMP, as well as CMP, was inhibited by free Mg, and only slightly inhibited by free ATP to extents much smaller than those seen in their 2'-deoxyribose-counterparts. Increasing amounts of ATP/Mg did not affect the phosphorylation of UMP, FUMP, and CMP. For the monophosphates of other nucleoside analogs, including ddC, L-ddC, and L-SddC, free Mg, free ATP, and excessive amount of ATP/Mg had enhancing, inhibitory, and
inhibitory effects on the phosphorylation, respectively. This suggested that D- or L-configuration and 3'-position were not determinants of this regulation.

Finally, the phosphorylation of monophosphates of several anticancer deoxycytidine analogs, such as ara-C, gemcitabine, and L-OddC, was regulated by ATP or Mg in a manner similar to the phosphorylation of dCMP.
DISCUSSION

After human UMP/CMP kinase was cloned and purified, the understanding of this enzyme has been advanced significantly in the past few years. It is now known that human UMP/CMP kinase is a member of nucleoside monophosphate (NMP) kinase family, and is highly homologous to adenylate kinase (AK) (Van Rompay et al, 1999; Yan and Tsai, 1999). Similar to UMP/CMP kinases of other species, human UMP/CMP kinase has 3 major functional domains, i.e. the nucleoside triphosphate binding glycine rich region, the NMP binding site, and LID domain (Van Rompay et al, 1999; Yan and Tsai, 1999). Despite similarities in amino acid sequence and structure, there are major differences in enzymatic characteristics among UMP/CMP kinases of different species. First, while UMP/CMP kinases of most eukaryotes, such as mammals and amoeba (Dictyostelium discoideum), show similar substrate specificity, yeast UMP/CMP kinase can phosphorylate AMP in addition to UMP and CMP (Muller-Dieckmann and Schulz, 1994 & 1995). Second, in contrast to eukaryotes whose UMP/CMP kinases represent a single enzyme phosphorylating UMP and CMP, bacterial CMP kinase and UMP kinase are two distinctive enzymes (Serina et al, 1995; Bucurenci et al, 1996; Briozzo et al, 1998). Another dramatic difference between bacterial CMP kinase and
eukaryotic UMP/CMP kinases is that bacterial CMP kinase can phosphorylate dCMP nearly as well as CMP (Serina et al, 1995; Bucurenci et al, 1996). The different kinetic characteristics and structure biology studies of UMP or CMP kinases from different species help delineate the molecular mechanisms responsible for substrate specificity and catalytic phosphorylation of this enzyme (Müller-Dieckmann and Schulz, 1994 & 1995; Serina et al, 1995; Wiesmüller et al, 1995; Bucurenci et al, 1996; Scheffzek et al, 1996; Briozzo et al, 1998; Hutter and Helms, 2000; Bertrand et al, 2002; Yu et al, 2003). For example, it has been shown that an approximate 40- amino acid residue insertion in the NMP binding domain is common in CMP kinases from bacteria (Serina et al, 1995; Bucurenci et al, 1996; Briozzo et al, 1998). In addition, the crystal structure and mutagenesis studies of *E. coli* CMP kinase showed that the serine101- residue located in this NMP- binding domain insertion plays a critical role in determining the preference for dCMP (Berstrand et al, 2002). It is worthy to note that previous structural studies of these non-mammalian UMP/CMP kinases did not reveal a dimer structure.

In this report, we provide kinetic data on human UMP/CMP kinase indicating that the kinetically active sites of this enzyme to interact with either dCMP or CMP should be different. The fact that there may be 2 distinct
binding sites for CMP or dCMP could be explained by 2 hypothetical models for the phosphorylation action of this enzyme. One possibility is the “asymmetric dimer” model, in which the enzyme is composed of two subunits, representing two catalytically active sites: one for dCMP (and most pyrimidine nucleoside analog monophosphates) and the other for CMP (as well as UMP and FUMP). Another possibility is that human UMP/CMP kinase exists in two different monomers: one is preferential for CMP phosphorylation and the other is preferential for dCMP phosphorylation (Fig.6). There might be certain equilibrium between the 2 hypothetic models. Interestingly, our data also indicate that binding of different substrates to the site other than their own catalytically active site does exist. For example, we showed that while CMP is a non-competitive inhibitor of dCMP phosphorylation, dCMP is a competitive inhibitor of CMP phosphorylation. This can be envisioned as dCMP interacts with the site which is catalytically active for CMP, in addition to its own active site.

Human UMP/CMP kinase is the only known enzyme to phosphorylate dCMP in human cells. However, given the facts that dCMP exists in a hundred-fold less amount than CMP or UMP in cells (Traut, 1994a) and that dCMP is phosphorylated by recombinant human UMP/CMP kinase with an
efficiency 100-fold less than UMP or CMP (Van Rompay et al, 1999; Liou et al, 2002; Pasti et al, 2003), it is almost impossible for dCMP to be phosphorylated in cells if all these naturally occurring pyrimidine monophosphates compete with each other at the same binding site of the enzyme. Similarly, the phosphorylation of pyrimidine nucleoside analog monophosphates, including ara-CMP, gemcitabine MP, and L-OddCMP, by human UMP/CMP kinase could also be very difficult in cells because the efficiency for these analog monophosphates to be phosphorylated by this enzyme is only comparable to or even less efficient than the phosphorylation of dCMP (Liou et al, 2002). Our proposed models, either asymmetric dimer or coexisting different monomers, indicate that it is difficult for CMP or UMP to completely inhibit dCMP phosphorylation due to the non-competitive nature of this inhibition. Furthermore, the physiological concentration of intracellular ATP is $3.1 \pm 1.7$ mM, and that of Mg is $1.1$ mM in free form and $8$ mM in complex form (Traut TW 1994). The concentration ranges that we studied were within the ranges mentioned above. In addition, the concentrations of nucleotides or metal ions are known to vary in different sub-cellular localizations due to compartmentalization or by different regulatory mechanisms. In this report, we showed that changing the concentrations of ATP and/or Mg would make
the enzyme more favorable in phosphorylating dCMP than CMP. This regulatory mechanism, as well as the non-competitive nature of CMP or UMP in inhibiting dCMP phosphorylation, provides a possible explanation how dCMP and other analog monophosphates can be phosphorylated in cells.

Many enzymes do exist as oligomers or dimers. The reversible dissociation and re-association of subunits of oligomers or dimers may lead to conformational change and provide possible regulatory mechanisms for enzyme activities (Traut, 1994b). Several enzymes in nucleotide metabolism are known to be dimers. For example, human thymidine monophosphate kinase (TMPK), another pyrimidine monophosphate kinase of cells, is known to be a homodimeric globular protein (Ostermann et al, 2000). Human deoxycytidine kinase (dCK), which is responsible for the phosphorylation of natural deoxynucleosides and numerous nucleoside analogs, has been shown to be a homodimeric globular protein, too (Sabini et al, 2003). In an early literature studying the pyrimidine monophosphate kinase of human leukemic cells, the authors used gel filtration chromatography and found out that some dCMP kinase activity resided in “aggregated” proteins, i.e. those proteins with an estimated molecular weight 2 to 3 fold higher than the molecular weight from the major activity peak (Hande and Chabner, 1978). Another early
report studying highly purified UMP kinase from rat liver demonstrated that the enzyme could be converted from a large molecular weight (MW) form (MW~57,000 dalton) to a low MW form (MW~17,000 dalton) by sulfhydryl reducing agents (Maness and Orengo, 1976). On the other hand, a recent report about the crystal structure of *E. coli* CMP kinase revealed 2 different binding modes for the enzyme when complexed with dCMP, ara-CMP, or ddCMP (Berstrand et al, 2002). The authors thus hypothesized that there were 2 kinds of molecules with different binding modes coexisting in the solution.

These indirect observations, although supportive of our hypothesis about human UMP/CMP kinase, do not differentiate either possibility. Our preliminary work employing Superdex™ 75 gel filtration (Amersham Biosciences Corp., Piscataway, NJ) found out that the recombinant human UMP/CMP kinase was eluted with a molecular weight estimated as 32,000 ~ 35,000 dalton (data not shown). This molecular weight is definitely larger than the known molecular weight of this protein (~ 20,000 dalton evaluated by SDS-polyacrylamide gel electrophoresis). Currently, we are undertaking cross-linker studies to verify our hypothesis.

How a change of ATP or Mg can affect CMP or dCMP phosphorylation reactions of human UMP/CMP kinase remains unanswered in this report.
The observation that an increase of free Mg and a reduction of ATP could suppress CMPK activity cannot be explained by the change of the kinetic parameters in phosphorylating CMP (Table 1). A possible mechanism is related to the substrate-inhibition effect of CMP on its own phosphorylation reaction. As reported in a previous report (Pasti et al, 2003), we also observed that recombinant human CMP/UMP kinase has a substrate-inhibition effect for UMP and CMP at concentrations higher than 0.1 ~ 0.2 mM, but not for dCMP. Our preliminary data suggest that when ATP/Mg is changed to conditions unfavorable to CMP phosphorylation (such as 0.1/5 mM), the substrate-inhibition effect by CMP would become much more pronounced (data not shown). However, the detailed biochemical basis for this regulation needs further investigation.

Based on the studies of human cancer cells, UMP/CMP kinase has been suggested to play a significant role in cancer biology and cancer therapy decades ago (Hande and Chabner et al, 1978; Scott and Wright, 1979). Recently, it has been suggested that human UMP/CMP kinase might be one of the novel mechanisms contributing to the clinical drug resistance to 5-fluorouracil and other nucleoside analog anticancer drugs (Banerjee et al, 2002). Our current studies provide a more complicated picture of this
enzyme with regards to the regulation of its kinase activity. Further understanding of the significance of this enzyme and its physiologic regulations may help develop new strategies for anticancer treatment in the future.
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human dCK suggests strategies to improve anticancer and antiviral

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FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1  Effect of free ATP (A), ATP/Mg (B), and free Mg (C) on the phosphorylation of CMP (left panel) or dCMP (right panel) by recombinant human UMP/CMP kinase. To test the effect of free ATP (A), the phosphorylation of CMP or dCMP was performed under various concentrations of ATP (1 to 8 mM) and a fixed concentration of Mg (2 mM). There was excessive free ATP in the reactions when ATP was more than 2 mM. For the effect of ATP/Mg (B), the reactions were performed with equal concentrations of ATP and Mg ranging from 0 to 8 mM. For the effect of free Mg (C), the reactions were undergone with various concentrations of Mg (0~ 8 mM) and a fixed concentration of ATP (2 mM). Therefore, the impact of free Mg could be seen when Mg was more than 2 mM. All the reactions were performed under substrate 1 mM, DTT 2 mM, and NaF 10 mM, at 37°C. The data are presented as mean with standard deviation from at least 3 independent experiments.

Fig. 2  Enzyme inhibition study of recombinant human UMP/CMP kinase: Inhibition of UMP phosphorylation by CMP. The reactions were performed with 0~ 100 µM of UMP in the presence of different concentrations
of CMP. All reaction mixtures contained 2 mM DTT and 10 mM NaF. The representative experiment shown here was performed when ATP and Mg were 0.1 and 2 mM, respectively (A). Lineweaver-Burk plot was processed to understand the pattern of enzyme inhibition (B). Re-plotting the slopes of Lineweaver-Burk plots versus inhibitor concentrations, the $K_i$ value of the inhibitor was then determined (left upper insertion of B).

**Fig. 3** Enzyme inhibition study of recombinant human UMP/CMP kinase: Inhibition of dCMP phosphorylation by CMP. The reactions were performed with 0~1000 µM of dCMP in the presence of different concentrations of CMP. All reaction mixtures contained 2 mM DTT and 10 mM NaF. The representative experiment shown here was performed when ATP and Mg were 0.1 and 2 mM, respectively (A). Lineweaver-Burk plot was processed to understand the pattern of enzyme inhibition (B). Re-plotting the slopes of Lineweaver-Burk plots versus inhibitor concentrations, the $K_i$ value of the inhibitor was then determined (left upper insertion of B).

**Fig. 4** Enzyme inhibition study of recombinant human UMP/CMP kinase: Inhibition of CMP phosphorylation by dCMP. The reactions were
performed with 0~ 80 µM of CMP in the presence of different concentrations of dCMP. All reaction mixtures contained 2 mM DTT and 10 mM NaF. The representative experiment shown here was performed when ATP and Mg were 2 and 2 mM, respectively (A). Lineweaver-Burk plot was processed to understand the pattern of enzyme inhibition (B). Re-plotting the slopes of Lineweaver-Burk plots versus inhibitor concentrations, the $K_i$ value of the inhibitor was then determined (left upper insertion of B).

**Fig. 5** Evaluation of the impact of free Mg2+, free ATP, and ATP/Mg in the phosphorylation of pyrimidine or analog monophosphates by recombinant human UMP/CMP kinase: Impact on CMP (A) and dCMP (B) phosphorylation served as an example. Two sets of ATP/Mg combinations were used in these experiments: one with Mg fixed to 2 mM, another with equal concentrations of ATP and Mg. For the former (Mg= 2 mM), the ATP/Mg concentrations were 0.5/2 mM, 2/2 mM, and 5/2 mM; for the latter(Mg// ATP), the combinations were 0.5/0.5 mM, 2/2 mM, and 5/5 mM. The reactions were performed at the same time with 2 mM DTT and 10 mM NaF at 37°C. The effect of free Mg was determined from the difference between ATP/Mg 0.5/0.5 mM and 0.5/2 mM. The impact of free ATP was
derived from the difference between ATP/Mg 5/2 mM and 2/2 mM. The effect of ATP/Mg was calculated from the difference between ATP/Mg 5/5 mM versus 2/2 mM. The impact on CMP and dCMP phosphorylation, derived from at least 3 independent experiments, is expressed in mean with standard deviation shown in (A) and (B), respectively.

Fig. 6 Models for human UMP/CMP kinase. Based on the data presented in this report, models for the action of recombinant human UMP/CMP kinase are proposed. Model (A) is “asymmetric dimer” model. Dimers, which are composed of 2 subunits, are subjected to different regulatory factors. The NMP binding region of one subunit represents the catalytically active site of CMP, UMP, and 5FUMP; the NMP binding region of the other subunit is the catalytically active site for dCMP, dUMP, 5FdUMP, and other pyrimidine analog monophosphates. Model (B) is the “coexisting 2 different monomers” model. Two monomers, which are functionally the same as the 2 subunits described in the “asymmetric dimer” model, coexist in the solution. There might be certain equilibrium between the 2 proposed models.
TABLE 1

Kinetic properties of recombinant human UMP/CMP kinase in phosphorylating dCMP or CMP under different ATP/Mg2+ combinations.

All reactions were performed at 37°C, using methods described in “Materials and Methods”. Km's were derived from Lineweaver-Burk plots. Vmax was calculated using the Michaelis-Menton equation: v= Vmax [S]/Km + [S].

Relative efficiency= Vmax/Km x 100%, relative to dCMP phosphorylation at ATP/Mg 8/8 mM. Values are presented as mean ± standard deviation from at least 3 independent experiments.

<table>
<thead>
<tr>
<th>Phosphate acceptor</th>
<th>ATP/Mg (mM)</th>
<th>Km (µM)</th>
<th>Vmax (µmol/min/mg protein)</th>
<th>Relative efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dCMP</td>
<td>0.1/2</td>
<td>404 ± 23</td>
<td>413 ± 19</td>
<td>492</td>
</tr>
<tr>
<td></td>
<td>0.5/2</td>
<td>526 ± 70</td>
<td>540 ± 100</td>
<td>495</td>
</tr>
<tr>
<td></td>
<td>2/2</td>
<td>906 ± 36</td>
<td>420 ± 30</td>
<td>223</td>
</tr>
<tr>
<td></td>
<td>8/8</td>
<td>1388 ± 4</td>
<td>288 ± 3</td>
<td>100</td>
</tr>
<tr>
<td>CMP</td>
<td>0.1/2</td>
<td>5 ± 2</td>
<td>513 ± 88</td>
<td>49400</td>
</tr>
<tr>
<td></td>
<td>0.5/2</td>
<td>N.D.²</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>2/2</td>
<td>15 ± 6</td>
<td>543 ± 42</td>
<td>17400</td>
</tr>
<tr>
<td></td>
<td>8/8</td>
<td>29 ± 11</td>
<td>620 ± 200</td>
<td>10300</td>
</tr>
</tbody>
</table>

² N.D.: Not done.
TABLE 2

Enzyme inhibition study of recombinant human UMP/CMP kinase:

Inhibition of UMP or dCMP phosphorylation by CMP. The enzyme inhibition studies were performed with either 0~100 µM of UMP or 0~1000 µM of dCMP in the presence of different concentrations of CMP. Reactions were performed with ATP/Mg concentrations either 0.1/2 mM or 2/2 mM, DTT 2 mM, and NaF 10 mM. The \( K_i \) values of CMP for inhibiting UMP or dCMP phosphorylation (\( K_{i_{CMP}} \) on UMPK or \( K_{i_{CMP}} \) dCMPK) were derived according to Fig. 2 and Fig. 3. The data are presented as mean with standard deviation from at least 3 separate experiments. The \( K_m \) values of CMP, as a reference to compare with \( K_i \) values, are from Table 1.

<table>
<thead>
<tr>
<th>ATP/Mg (mM)</th>
<th>( K_m_{CMP} ) (µM)</th>
<th>( K_i ) on UMPK (µM)</th>
<th>( K_i ) on dCMPK (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1/2</td>
<td>5 ± 2</td>
<td>7 ± 2</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>2/2</td>
<td>15 ± 6</td>
<td>12 ± 3</td>
<td>91 ± 19</td>
</tr>
</tbody>
</table>
TABLE 3

Enzyme inhibition study of recombinant human UMP/CMP kinase:

Inhibition of CMP phosphorylation by dCMP. The enzyme inhibition studies were performed with 0~80 µM of CMP in the presence of different concentrations of dCMP. Reactions were performed with ATP/Mg concentrations either 0.1/2 mM or 2/2 mM, DTT 2 mM, and NaF 10 mM. The $K_i$ values of dCMP for inhibiting CMP phosphorylation ($K_{idCMP}$ on CMPK) were derived according to Fig.4. The data are presented as mean with standard deviation from at least 3 separate experiments. The $K_m$ values of dCMP, as a reference to compare with $K_i$ values, are from Table 1.

<table>
<thead>
<tr>
<th>ATP/Mg (mM)</th>
<th>$K_m$ dCMP (µM)</th>
<th>$K_i$ on CMPK (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1/2</td>
<td>406 ± 23</td>
<td>440 ± 70</td>
</tr>
<tr>
<td>2/2</td>
<td>906 ± 36</td>
<td>801 ± 128</td>
</tr>
</tbody>
</table>
TABLE 4

Effect of free Mg, free ATP, and ATP/Mg in the phosphorylation of pyrimidine nucleoside and analog monophosphates by recombinant human UMP/CMP kinase. The reactions were performed using 2 sets of ATP/Mg concentration combinations: Mg fixed to 2 mM and equal concentrations of ATP/Mg. As demonstrated in Fig. 5, the difference between ATP/Mg 0.5/2 mM and 0.5/0.5 mM, 5/2 mM and 2/2 mM, 5/5 mM and 2/2 mM revealed the effect of free Mg, free ATP, and ATP/Mg, respectively. All reactions were performed when the concentration of phosphate acceptor was 1 mM, except for gemcitabine-MP (dFdCMP), ddCMP, L-ddCMP, L-OddCMP, and L-SddCMP. Values are presented as mean ± standard deviation from at least 2 independent experiments.

<table>
<thead>
<tr>
<th>Nucleoside or analog monophosphates</th>
<th>Effect of free Mg</th>
<th>Effect of free ATP</th>
<th>Effect of ATP/Mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP</td>
<td>↓ 29.2 ± 7.1</td>
<td>↑ 4.3 ± 9</td>
<td>↑ 10.2 ± 8.0</td>
</tr>
<tr>
<td>dCMP</td>
<td>↑ 70.2 ± 17.9</td>
<td>↓ 73.6 ± 3.6</td>
<td>↓ 29.1 ± 2.7</td>
</tr>
<tr>
<td>UMP</td>
<td>↓ 42.0 ± 8.4</td>
<td>↓ 26.6 ± 11.7</td>
<td>↑ 7.9 ± 17.6</td>
</tr>
<tr>
<td>dUMP</td>
<td>↑ 116.6 ± 57.9</td>
<td>↓ 82.2 ± 17.1</td>
<td>↓ 44.5 ± 7.4</td>
</tr>
<tr>
<td>FUMP</td>
<td>↓ 19.6 ± 5.3</td>
<td>↓ 30.0 ± 14.4</td>
<td>↑ 3.2 ± 2.2</td>
</tr>
<tr>
<td>FdUMP</td>
<td>↑ 54.4 ± 35.7</td>
<td>↓ 80.1 ± 1.5</td>
<td>↓ 27 ± 12.5</td>
</tr>
<tr>
<td>AraCMP</td>
<td>↑ 16.5 ± 1.7</td>
<td>↓ 66.1 ± 1.3</td>
<td>↓ 22.0 ± 6.1</td>
</tr>
<tr>
<td>dFdCMP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>↑ 75.4 ± 3.4</td>
<td>↓ 64.6 ± 1.8</td>
<td>↓ 20.0 ± 3.9</td>
</tr>
<tr>
<td>ddCMP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>↑ 83.7 ±11.2</td>
<td>↓ 38.2 ± 13.2</td>
<td>↓ 21.0 ± 10.8</td>
</tr>
<tr>
<td>L-ddCMP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>↑ 50.4 ±7.2</td>
<td>↓ 50.8 ± 11.6</td>
<td>↓ 16.5 ± 11.6</td>
</tr>
<tr>
<td>L-OddCMP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>↑ 40.0 ± 5.5</td>
<td>↓ 39.8 ± 23.2</td>
<td>↓ 10.8 ± 3.4</td>
</tr>
<tr>
<td>L-SddCMP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>↑ 60.1 ± 10.4</td>
<td>↓ 56.1 ± 5.6</td>
<td>↓ 12.7 ± 3.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> The concentration of dFdCMP was 0.2 mM.

<sup>b</sup> The concentration of ddCMP, L-ddCMP, L-OddCMP, and L-SddCMP was 0.5 mM.
Fig. 1

(A) CMPK activity as a function of ATP concentration.

(B) CMPK activity as a function of Mg2+ concentration.

(C) dCMPK activity as a function of ATP concentration.

(dCMPK activity as a function of Mg2+ concentration.)
Fig. 2

(A)

![Graph showing enzyme activity versus UMP concentration for different CMP concentrations.](image)

- Enzyme activity (µmol/min/mg protein) vs. UMP (µM) for CMP concentrations of 0 µM, 15 µM, and 30 µM.

(B)

![Graph showing the double reciprocal plot for enzyme activity versus UMP concentration.](image)

- The double reciprocal plot illustrates the enzyme velocity (1/µmol/min) against the inverse of UMP concentration (1/[UMP] µM).

- The graphs in (B) are used to determine the kinetic parameters of the enzyme.

- The data points are fitted to the lines, and the slopes and intercepts are used to calculate the kinetic constants.

- The R² value for the graph is 0.9824, indicating a strong correlation.

- The calculated parameters are:
  - Kinase activity
  - 1/UMP (µM)
  - 1/[Kinase activity (µmol/min/mg protein)]

- The graphs are used to determine the enzyme's sensitivity to substrate and inhibitor concentrations.
Fig. 3

(A)

Kinase activity (μmol/min/mg protein) vs. dCMP (μM)

- CMP = 0 μM
- CMP = 5 μM
- CMP = 15 μM
- CMP = 30 μM

(B)

1/[Kinase activity (μmol/min/mg protein)] vs. 1/[dCMP (μM)]

- CMP = 0 μM
- CMP = 5 μM
- CMP = 15 μM
- CMP = 30 μM

Ki = 30 μM
r² = 0.9812

Slope = Km / (Vmax x Ki)
Fig. 4

(A)

Kinase activity (µmol/min/mg protein) vs. CMP (µM)

- ■ dCMP = 0 µM
- ▲ dCMP = 500 µM
- ▼ dCMP = 1000 µM

(B)

1/[CMP (µM)] vs. 1/[Kinase activity (µmol/min/mg protein)]

- ■ dCMP = 0 µM
- ▲ dCMP = 500 µM
- ▼ dCMP = 1000 µM

Kid CMP = 682 µM

r² = 0.9904

Slope [= Km / (Vmax x Ki)]
Fig. 5

(A)

(B)
Fig. 6

- ▲ Active site for dCMP and deoxypyrimidine nucleoside analog-monophosphates
- ■ Active site for CMP, UMP, and FUMP