0026-895X/05/6703-806–814$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2005 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 67:806–814, 2005

Vol. 67, No. 3
6098/1193670
Printed in U.S.A.

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

Chih-Hung Hsu, Jieh-Yuan Liou, Ginger E. Dutschman, and Yung-Chi Cheng

Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut (C.-H.H., G.E.D., Y.-C.C.); Department of Oncology, National Taiwan University Hospital, Taipei, Taiwan (C.-H.H); and Division of Cancer Research, National Health Research Institutes, Taipei, Taiwan (J.-Y.L.)

Received August 12, 2004; accepted November 18, 2004

ABSTRACT

Human UMP/CMP kinase (cytidylate kinase; EC 2.7.4.14) is responsible for phosphorylation of CMP, UMP, and deoxycytidine monophosphate (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 reevaluated 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 were important regulators of the kinase activities of this enzyme. Free magnesium enhanced dCMPK activity but inhibited CMPK activity. Free ATP or excess ATP/magnesium, on the other hand, inhibited dCMPK but not CMPK reactions. The differential regulation of dCMPK versus CMPK activities by ATP or magnesium was also seen in other 2′-deoxypyrimidine analog monophosphates (deoxyuridine monophosphate, 5-fluorodeoxyuridine monophosphate, 1-β-D-arabinofuranosylcytosine monophosphate, and gemcitabine monophosphate) versus their ribose-counterparts (UMP and 5-fluorouridine monophosphate), 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 noncompetitive 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.

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). A conditional lethal mutant isolated from *Saccharomyces cerevisiae* was recently 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 anticancer and antiviral agents (Cheng, 2001; Galmarini et al., 2001, 2002). For example, 1-β-D-arabinofuranosylcytosine 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-dioxaolanecytidine (L-OddC), a deoxycytidine analog with an unnatural L-configuration, is currently under active clinical investigations because of promising antitumor 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). Furthermore, 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 and anti-human

This work was supported by National Institutes of Health grants R01-CA63477 and R01-AI38204 (to Y.-C.C.). Y.-C.C. is a fellow of the National Foundation for Cancer Research.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.104.006098.

ABBREVIATIONS: dCMP, deoxycytidine monophosphate; ara-CMP, 1-β-D-arabino furanosyl cytosine monophosphate; L-SSdC or 3-TC, β-L-2′, 3′-dideoxy-3′-thiacytidine; ddC, β-D-2′, 3′-dideoxycytidine; dUMP, deoxyuridine monophosphate; FdUMP, 5-fluorodeoxyuridine monophosphate; 5FUMP, 5-fluorouridine monophosphate; L-ddC, L-2′, 3′-dideoxycytidine; L-OddC, β-L-dioxaolanecytidine; DTT, dithiothreitol; NMP, nucleoside monophosphate; CMPK, CMP kinase; dCMPK, deoxy-CMP kinase.

hepatitis B virus 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).

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 with 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, 1994b), the kinetic properties of recombinant human UMP/CMP kinase in vitro raise a question of how dCMP can be phosphorylated by this enzyme inside cells. It also implies that other cellular mechanisms that 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 unidentified cellular dCMP kinases, have yet to be proven (Liou et al., 2002).

In this report, we started with a careful reevaluation of the kinetic properties of human recombinant UMP/CMP kinase and found that the phosphorylation of CMP or dCMP by this enzyme was regulated by ATP and magnesium very differently. We went on to demonstrate that different concentrations of ATP and magnesium 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 noncompetitive 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 (St. Louis, MO). 5-Fluorouridine monophosphate (5FUMP), [5-^{3}H]-CMP, [5-^{3}H]dCMP, [5-^{3}H]UMP, and [5-^{3}H]dUMP were purchased from Moravek Biochemicals (Brea, CA). The monophosphates of other nucleoside analog, including gemcitabine, ddC, L-2′, 3′-dideoxycytidine (L-ddC), L-SddC, and L-OddC, were synthesized and purified according to the procedures published previously 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, Madison, WI), the protein was expressed with an N-terminal His-tag/thrombin configuration. The details of expression, purification, and thrombin cutting were reported previously (Liou et al., 2002). In short, BL21-Gold (DE3)-competent *Escherichia coli* cells (Stratagene, 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, 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, Clifton, NJ) assay and a high-performance liquid chromatography 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 Tris-HCl, pH 7.5, 10 mM NaF, and 2 mM DTT, supplemented with creatine phosphate and creatine kinase for regeneration of ATP in a total volume of 75 μl for each reaction. The reaction was performed at 37°C for 20 to 120 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 three 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. To improve the detection of ^{3}H-labeled radioactive nucleotides, compounds were eluted from the discs by incubation with 1 ml of 0.1 N HCl containing 2 M NaCl for 20 min before reading by a scintillation counter (Beckman Coulter Inc., Fullerton, CA). The enzyme activities were expressed as nanomoles per minute per milligram of 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 high-performance liquid chromatography (Shimadzu America, 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 Magnesium Regulate the CMPK or dCMPK Activities of Recombinant Human UMP/CMP Kinase Very Differently. The effects of ATP, ATP/magnesium, and magnesium 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 ATP, in the presence of 2 mM magnesium. 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 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/magnesium (in equal concentrations) on CMPK and dCMPK activities of recombinant human UMP/CMP kinase, whereas both CMPK and dCMPK activities achieved maximal when ATP/magnesium was ~1 to 2 mM, further increases of ATP/magnesium significantly suppressed dCMPK activity, but it had no inhibition on CMPK activity (Fig. 1B).

The requirement for magnesium in the kinase activity was much more stringent in dCMPK reaction than in CMPK reaction. Under 0 mM magnesium, 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 magnesium was 2 mM, and further increases of magnesium concentration resulted in a mild dose-dependent inhibition (Fig. 1C). In contrast, the optimum of dCMPK reaction was at 4 to 6 mM of magnesium; i.e.,

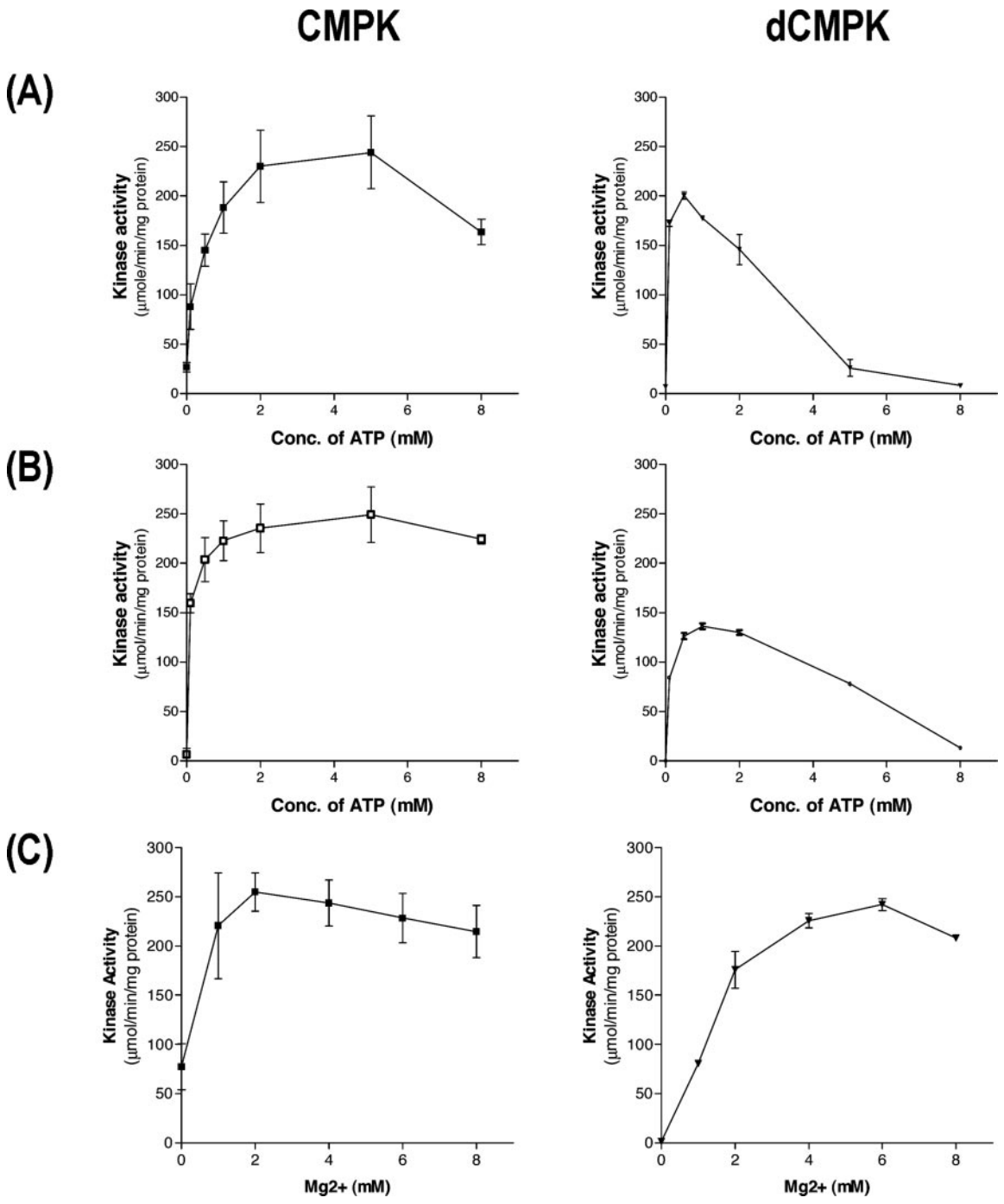

Fig. 1. Effect of free ATP (A), ATP/magnesium (B), and free magnesium (C) on the phosphorylation of CMP (left) or dCMP (right) by recombinant human UMP/CMP kinase. To test the effect of free ATP (A), the phosphorylation of CMP or dCMP was performed under various concentrations of ATP (1–8 mM) and a fixed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

there was 2 to 4 mM free magnesium in reactions. Further increase of free magnesium might have some inhibitory effect on dCMPK activity (Fig. 1C).

Change of ATP/Magnesium 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/magnesium have a significant inhibition on dCMPK activity but not on CMPK activity of recombinant human UMP/CMP kinase, and free magnesium has enhancing and suppressing effects on dCMPK activity and CMPK activity, respectively. We wondered whether an increase of free magnesium 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/magnesium (Table 1).

When ATP/magnesium concentrations were changed from 8/8 mM, which was very 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 values 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 also was increased for 4.9-fold by changing ATP/magnesium from 8/8 mM to 0.1/2 mM (Table 1). In other words, the enzyme kinetic studies demonstrated that the change of ATP/magnesium 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 magnesium concentrations could not be the explanation for what we have observed on the different regulatory effects of ATP and magnesium in phosphorylating dCMP or CMP.

Enzyme Inhibition Studies Show That CMP Noncompetitively Inhibited dCMP Phosphorylation by Recombinant Human UMP/CMP Kinase. The different regulation patterns by ATP and magnesium 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 with 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 noncompetitive 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). These data suggest that CMP or UMP inhibits recombinant protein from phosphorylating dCMP through sites other than the site responsible for its own binding site. It is interesting that 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 magnesium. Because 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 magnesium in a similar manner as the naturally occurring pyrimidine monophosphates. Using an approach shown in Fig. 5, we evaluated the modulatory effects of free ATP, free magnesium, and ATP/magnesium by comparing the differences of phosphorylation rates from reaction conditions with ATP/magnesium 5/2 versus 2/2 mM, 0.5/2 versus 0.5/0.5 mM, and 5/5 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 magnesium were generally related to the 2′ position of ribose. The phosphorylation of dUMP and FdUMP was enhanced by free magnesium, and suppressed by free ATP or excessive amounts of ATP/magnesium, in a pattern similar to dCMP phosphorylation (Fig. 5). On the other hand, phosphorylation of UMP or 5FUMP, as well as CMP, was inhibited by free magnesium and only slightly inhibited by free ATP to extents much smaller than those seen in their 2′-deoxyribose counterparts. Increasing amounts of ATP/magnesium did not affect the phosphorylation of UMP, 5FUMP, and CMP. For the monophosphates of other nucleoside analogs, including ddC,

TABLE 1

Kinetic properties of recombinant human UMP/CMP kinase in phosphorylating dCMP or CMP under different ATP/Mg^{2+} combinations

All reactions were performed at 37°C, using methods described under *Materials and Methods*. K_m values were derived from Lineweaver-Burk plots. V_{max} was calculated using the Michaelis-Menton equation: $v = V_{max}[S]/K_m + [S]$. Relative efficiency = $V_{max}/K_m \times 100\%$, relative to dCMP phosphorylation at ATP/Mg 8/8 mM. Values are presented as mean ± S.D. from at least three independent experiments.

Phosphate Acceptor	ATP/Mg	Kinetic Parameters		
		K_m	V_{max}	Relative Efficiency
	mM	*μM*	*μmol/min/mg of protein*	*%*
dCMP	0.1/2	404 ± 23	413 ± 19	492
	0.5/2	526 ± 70	540 ± 100	495
	2/2	906 ± 36	420 ± 30	223
	8/8	1388 ± 4	288 ± 3	100
CMP	0.1/2	5 ± 2	513 ± 88	49,400
	0.5/2	N.D.	N.D.	N.D.
	2/2	15 ± 6	543 ± 42	17,400
	8/8	29 ± 11	620 ± 200	10,300

N.D, not done.

L-ddC, and L-SddC, free magnesium, free ATP, and excessive amount of ATP/magnesium 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 1-β-D-arabinofuranosylcytosine, gemcitabine, and L-OddC, was regulated by ATP or magnesium in a manner similar to the phosphorylation of dCMP.

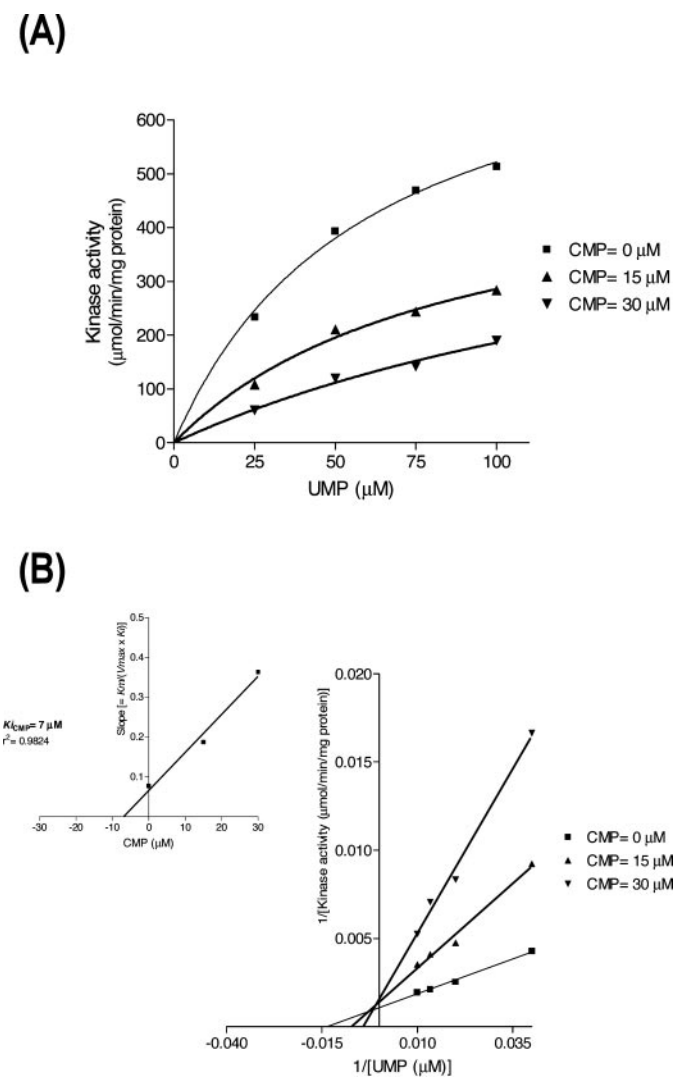

Fig. 2. Enzyme inhibition study of recombinant human UMP/CMP kinase. Inhibition of UMP phosphorylation by CMP. The reactions were performed with 0 to 100 μM UMP in the presence of different concentrations of CMP. All reaction mixtures contained 2 mM DTT and 10 mM NaF. The representative experiment shown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

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 to 100 μM of UMP or 0 to 1000 μM of dCMP in the presence of different concentrations of CMP. Reactions were performed with ATP/Mg concentrations either 0.1/2 or 2/2 mM, 2 mM DTT, and 10 mM NaF. 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 Figs. 2 and 3. The data are presented as mean with S.D. from at least three separate experiments. The K_m values of CMP, as a reference to compare with K_i values, are from Table 1.

ATP/Mg	$K_{m\ CMP}$	K_i on UMPK	K_i on dCMPK
		μM	
0.1/2 mM	5 ± 2	7 ± 2	21 ± 2
2/2 mM	15 ± 6	12 ± 3	91 ± 19

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 (Van Rompay et al., 1999; Yan and Tsai, 1999). Similar to UMP/CMP kinases of other species, human UMP/CMP kinase has three major functional domains, i.e., the nucleoside triphosphate binding glycine-rich region, the NMP bind-

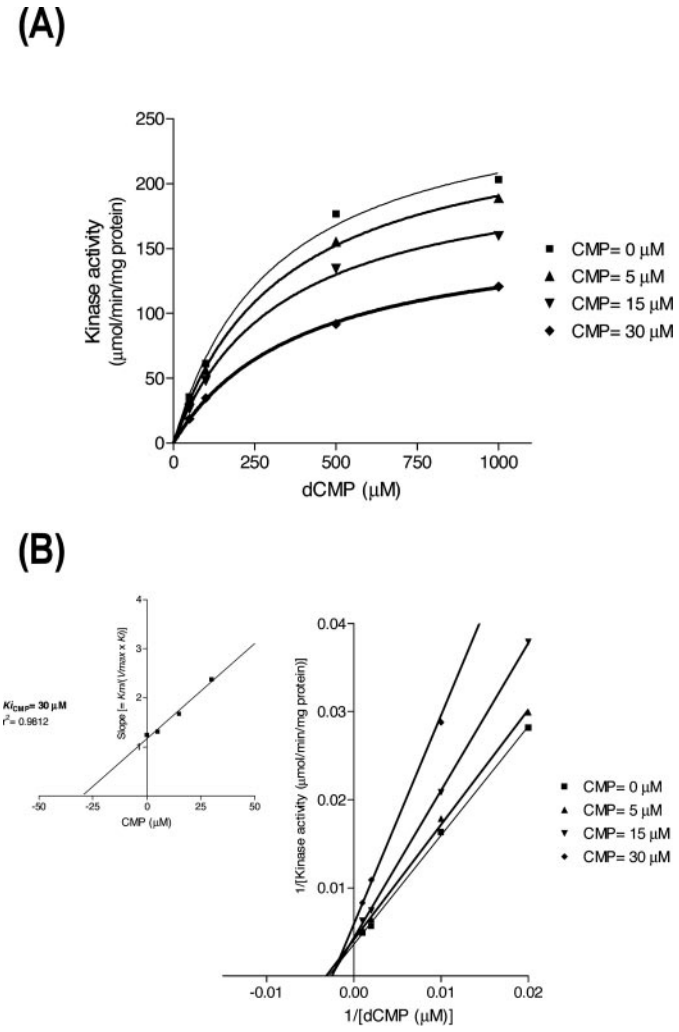

Fig. 3. Enzyme inhibition study of recombinant human UMP/CMP kinase. Inhibition of dCMP phosphorylation by CMP. The reactions were performed with 0 to 1000 μM dCMP in the presence of different concentrations of CMP. All reaction mixtures contained 2 mM DTT and 10 mM NaF. The representative experiment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

ing 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, although 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 cata-

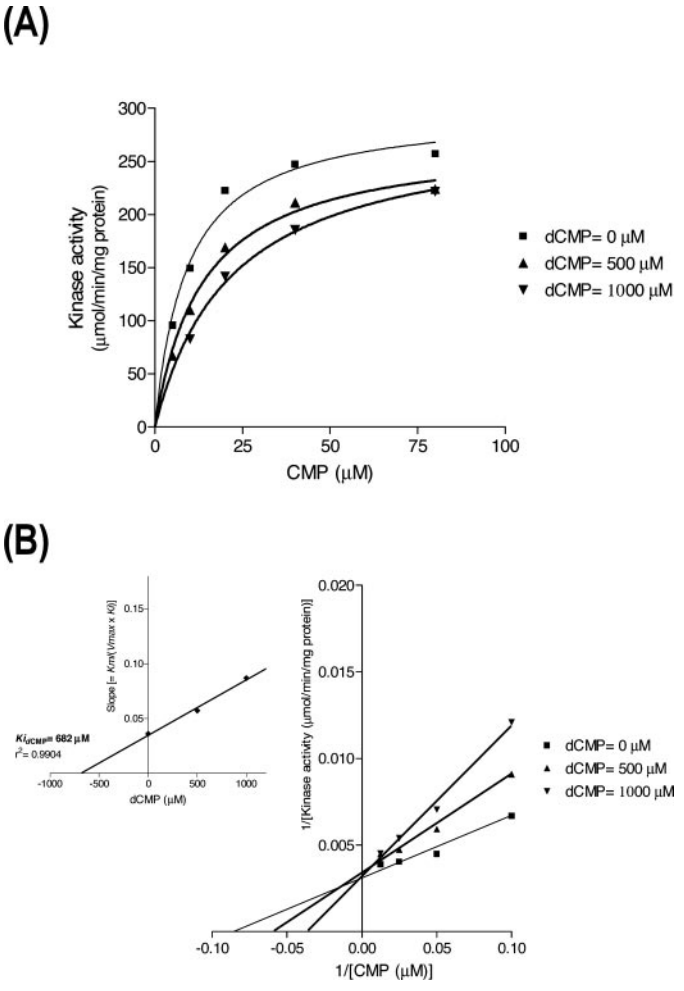

Fig. 4. Enzyme inhibition study of recombinant human UMP/CMP kinase. Inhibition of CMP phosphorylation by dCMP. The reactions were performed with 0 to 80 μM CMP in the presence of different concentrations of dCMP. All reaction mixtures contained 2 mM DTT and 10 mM NaF. The representative experiment shown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

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 to 80 μM of CMP in the presence of different concentrations of dCMP. Reactions were performed with ATP/Mg concentrations either 0.1/2 or 2/2 mM, 2 mM DTT, and 10 mM NaF . The K_i values of dCMP for inhibiting CMP phosphorylation ($K_{i\ dCMP}$ on CMPK) were derived according to Fig. 4. The data are presented as mean with S.D. from at least three separate experiments. The K_m values of dCMP, as a reference to compare with K_i values, are from Table 1.

ATP/Mg	$K_{m\ dCMP}$	K_i on CMPK
	μM	
0.1/2 mM	406 ± 23	440 ± 70
2/2 mM	906 ± 36	801 ± 128

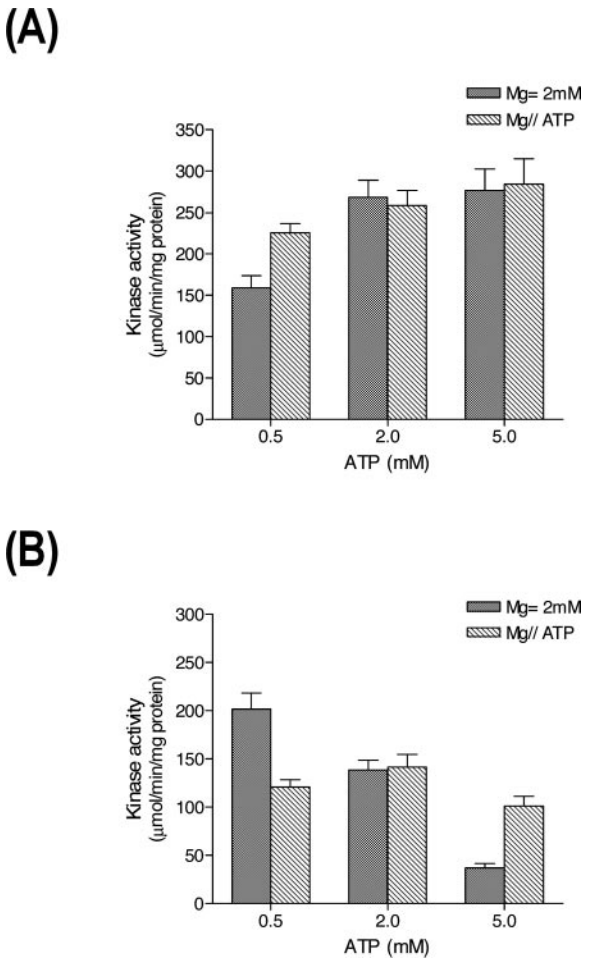

Fig. 5. Evaluation of the impact of free Mg^{2+}, free ATP, and ATP/magnesium in the phosphorylation of pyrimidine or analog monophosphates by recombinant human UMP/CMP kinase. Impact on CMP (A) and dCMP (B) phosphorylation served as an example. Two sets of ATP/magnesium combinations were used in these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

lytic phosphorylation of this enzyme (Muller-Dieckmann and Schulz, 1994, 1995; Serina et al., 1995; Wiesmuller 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 (Bertrand et al., 2002). It is noteworthy that previous structural studies of these nonmammalian 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. There may be two distinct binding sites for CMP or dCMP, which could be explained by two 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 5FUMP). 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 two hypothetic models. It is interesting that 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 although CMP is a noncompetitive inhibitor of dCMP phosphorylation, dCMP is a competitive inhibitor of CMP phosphorylation. This can be envisioned as dCMP interacts with the site that 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 fact that dCMP exists in a 100-fold less amount than CMP or UMP in cells (Traut, 1994b) 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. Likewise, the phosphorylation of pyrimidine nucleoside analog monophosphates, including ara-CMP, gemcitabine monophosphate, 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 with 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 because of the noncompetitive nature of this inhibition. Furthermore, the physiological concentration of intracellular ATP is 3.1 ± 1.7 mM and that of magnesium is 1.1 mM in free form and 8 mM in complex form (Traut, 1994b). 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 subcellular localizations because of compartmentalization or different regulatory mechanisms. In this report, we showed that changing the concentrations of ATP and/or magnesium would make the enzyme more favorable in phosphorylating dCMP than CMP. This regulatory mechanism, as well as the noncompetitive nature of CMP or UMP in inhibiting dCMP phosphorylation, provides a possible explanation of how dCMP and other analog monophosphates can be phosphorylated in cells.

Many enzymes do exist as oligomers or dimers. The reversible dissociation and reassociation of subunits of oligomers or dimers may lead to conformational change and provide possible regulatory mechanisms for enzyme activities (Traut, 1994a). Several enzymes in nucleotide metabolism are known to be dimers. For example, human thymidine monophosphate kinase, another pyrimidine monophosphate ki-

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 two 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 two independent experiments.

Nucleoside or Analog Monophosphates	Percentage of increase (↑) or inhibition (↓) (%)		
	Effect of Free Mg	Effect of Free ATP	Effect of ATP/Mg
CMP	↓ 29.2 ± 7.1	↑ 4.3 ± 9	↑ 10.2 ± 8.0
dCMP	↑ 70.2 ± 17.9	↓ 73.6 ± 3.6	↓ 29.1 ± 2.7
UMP	↓ 42.0 ± 8.4	↓ 26.6 ± 11.7	↑ 7.9 ± 17.6
dUMP	↑ 116.6 ± 57.9	↓ 82.2 ± 17.1	↓ 44.5 ± 7.4
FUMP	↓ 19.6 ± 5.3	↓ 30.0 ± 14.4	↑ 3.2 ± 2.2
FdUMP	↑ 54.4 ± 35.7	↓ 80.1 ± 1.5	↓ 27 ± 12.5
AraCMP	↑ 16.5 ± 1.7	↓ 66.1 ± 1.3	↓ 22.0 ± 6.1
dFdCMP[a]	↑ 75.4 ± 3.4	↓ 64.6 ± 1.8	↓ 20.0 ± 3.9
ddCMP[b]	↑ 83.7 ± 11.2	↓ 38.2 ± 13.2	↓ 21.0 ± 10.8
L-ddCMP[b]	↑ 50.4 ± 7.2	↓ 50.8 ± 11.6	↓ 16.5 ± 11.6
L-OddCMP[b]	↑ 40.0 ± 5.5	↓ 39.8 ± 23.2	↓ 10.8 ± 3.4
L-SddCMP[b]	↑ 60.1 ± 10.4	↓ 56.1 ± 5.6	↓ 12.7 ± 3.4

[a] The concentration of dFdCMP was 0.2 mM.
[b] The concentration of ddCMP, L-ddCMP, L-OddCMP, and L-SddCMP was 0.5 mM.

nase of cells, is known to be a homodimeric globular protein (Ostermann et al., 2000). Human deoxycytidine kinase, 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 mass 2- to 3-fold higher than the molecular mass 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 mass form (~57,000 Da) to a low molecular mass form (~17,000 Da) 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 two different binding modes for the enzyme when complexed with dCMP, ara-CMP, or ddCMP (Bertrand et al., 2002). The authors thus hypothesized that there were two 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 using Superdex 75 gel filtration (Amersham Biosciences Inc., Piscataway, NJ) found that the recombinant human UMP/CMP kinase was eluted with a molecular mass estimated as ~32,000 to 35,000 Da (data not shown). This molecular mass is definitely larger than the known molecular mass of this protein (~20,000 Da evaluated by SDS-polyacrylamide gel electrophoresis). We are currently undertaking cross-linker studies to verify our hypothesis.

How a change of ATP or magnesium can affect CMP or dCMP phosphorylation reactions of human UMP/CMP kinase remains unanswered in this report. The observation that an increase of free magnesium 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 to 0.2 mM, but not for dCMP. Our preliminary data suggest that when ATP/magnesium 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, 1978; Scott and Wright, 1979). It has recently 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.

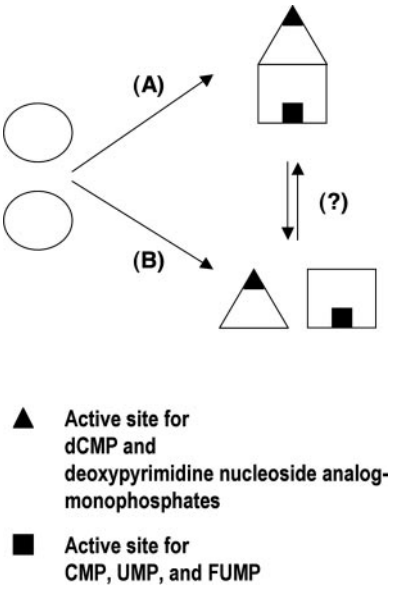

Fig. 6. Models for human UMP/CMP kinase. Based on the data presented in this report, models for the action of recombinant human UMP/CMP kinase are proposed. Model A is "asymmetric dimer" model. Dimers, which are composed of two subunits, are subjected to different regulatory factors. The NMP binding 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 two different monomers" model. Two monomers, which are functionally the same as the two subunits described in the "asymmetric dimer" model, coexist in the solution. There might be certain equilibrium between the two proposed models.

Acknowledgments

We thank Dr. Guangwei Yang for the discussion and the assistance in gel filtration studies.

References

Banerjee D, Mayer-Kuckuk P, Capiaux G, Budak-Alpdogan T, Gorlick R, and Bertino JR (2002) Novel aspects of resistance to drugs targeted to dihydrofolate reductase and thymidylate synthase. *Biochim Biophys Acta* **1587:**164–173.

Bertrand T, Briozzo P, Assairi L, Ofiteru A, Bucurenci N, Munier-Lehmann H, Golinelli-Pimpaneau B, Barzu O, and Gilles AM (2002) Sugar specificity of bacterial CMP kinases as revealed by crystal structures and mutagenesis of *Escherichia coli* enzyme. *J Mol Biol* **315:**1099–1110.

Briozzo P, Golinelli-Pimpaneau B, Gilles AM, Gaucher JF, Burlacu-Miron S, Sakamoto H, Janin J, and Barzu O (1998) Structures of *Escherichia coli* CMP kinase alone and in complex with CDP: a new fold of the nucleoside monophosphate binding domain and insights into cytosine nucleotide specificity. *Structure* **6:**1517–1527.

Bucurenci N, Sakamoto H, Briozzo P, Palibroda N, Serina L, Sarfati RS, Labesse G, Briand G, Danchin A, Barzu O, et al. (1996) CMP kinase from *Escherichia coli* is structurally related to other nucleoside monophosphate kinases. *J Biol Chem* **271:**2856–2862.

Cheng YC (2001) Potential use of antiviral L(−)nucleoside analogues for the prevention or treatment of viral associated cancers. *Cancer Lett* **162 (Suppl):**S33–S37.

Cheng YC and Prusoff WH (1974) A new rapid assay for measuring deoxycytidylate- and deoxythymidylate-kinase activities. *Anal Biochem* **60:**545–550.

Galmarini CM, Mackey JR, and Dumontet C (2001) Nucleoside analogues: mechanisms of drug resistance and reversal strategies. *Leukemia* **15:**875–890.

Galmarini CM, Mackey JR, and Dumontet C (2002) Nucleoside analogues and nucleobases in cancer treatment. *Lancet Oncol* **3:**415–424.

Grant S (1998) Ara-C: cellular and molecular pharmacology. *Adv Cancer Res* **72:**197–233.

Grove KL and Cheng YC (1996) Uptake and metabolism of the new anticancer compound beta-L-(−)-dioxolane-cytidine in human prostate carcinoma DU-145 cells. *Cancer Res* **56:**4187–4191.

Grove KL, Guo X, Liu SH, Gao Z, Chu CK, and Cheng YC (1995) Anticancer activity of beta-L-dioxolane-cytidine, a novel nucleoside analogue with the unnatural L configuration. *Cancer Res* **55:**3008–3011.

Hande KR and Chabner BA (1978) Pyrimidine nucleoside monophosphate kinase from human leukemic blast cells. *Cancer Res* **38:**579–585.
Hui YF and Reitz J (1997) Gemcitabine: a cytidine analogue active against solid tumors. *Am J Health Syst Pharm* **54:**162–170.
Hutter MC and Helms V (2000) Phosphoryl transfer by a concerted reaction mechanism in UMP/CMP-kinase. *Protein Sci* **9:**2225–2231.
Krishnan P, Fu Q, Lam W, Liou JY, Dutschman G, and Cheng YC (2002) Phosphorylation of pyrimidine deoxynucleoside analog diphosphates: selective phosphorylation of L-nucleoside analog diphosphates by 3-phosphoglycerate kinase. *J Biol Chem* **277:**5453–5459.
Liljelund P and Lacroute F (1986) Genetic characterization and isolation of the *Saccharomyces cerevisiae* gene coding for uridine monophosphokinase. *Mol Gen Genet* **205:**74–81.
Liou JY, Dutschman GE, Lam W, Jiang Z, and Cheng YC (2002) Characterization of human UMP/CMP kinase and its phosphorylation of D- and L-form deoxycytidine analogue monophosphates. *Cancer Res* **62:**1624–1631.
Maness P and Orengo A (1976) Activation of rat liver pyrimidine nucleoside monophosphate kinase. *Biochim Biophys Acta* **429:**182–190.
Muller-Dieckmann HJ and Schulz GE (1994) The structure of uridylate kinase with its substrates, showing the transition state geometry. *J Mol Biol* **236:**361–367.
Muller-Dieckmann HJ and Schulz GE (1995) Substrate specificity and assembly of the catalytic center derived from two structures of ligated uridylate kinase. *J Mol Biol* **246:**522–530.
Noble S and Goa KL (1997) Gemcitabine. A review of its pharmacology and clinical potential in non-small cell lung cancer and pancreatic cancer. *Drugs* **54:**447–472.
Ostermann N, Schlichting I, Brundiers R, Konrad M, Reinstein J, Veit T, Goody RS, and Lavie A (2000) Insights into the phosphoryltransfer mechanism of human thymidylate kinase gained from crystal structures of enzyme complexes along the reaction coordinate. *Structure* **8:**629–642.
Pasti C, Gallois-Montbrun S, Munier-Lehmann H, Veron M, Gilles AM, and Deville-Bonne D (2003) Reaction of human UMP-CMP kinase with natural and analog substrates. *Eur J Biochem* **270:**1784–1790.
Pearman AT, Castro-Faria-Neto HC, McIntyre TM, Prescott SM, and Stafforini DM (2001) Characterization of human UMP-CMP kinase enzymatic activity and 5′ untranslated region. *Life Sci* **69:**2361–2370.
Ruth JL and Cheng YC (1981) Nucleoside analogues with clinical potential in antivirus chemotherapy. The effect of several thymidine and 2′-deoxycytidine analogue 5′-triphosphates on purified human (α,β) and herpes simplex virus (types 1, 2) DNA polymerases. *Mol Pharmacol* **20:**415–422.
Sabini E, Ort S, Monnerjahn C, Konrad M, and Lavie A (2003) Structure of human dCK suggests strategies to improve anticancer and antiviral therapy. *Nat Struct Biol* **10:**513–519.
Scheffzek K, Kliche W, Wiesmuller L, and Reinstein J (1996) Crystal structure of the complex of UMP/CMP kinase from *Dictyostelium discoideum* and the bisubstrate inhibitor P1-(5′-adenosyl) P5-(5′-uridyl) pentaphosphate (UP5A) and Mg^{2+} at 2.2 A: implications for water-mediated specificity. *Biochemistry* **35:**9716–9727.
Scott EM and Wright RC (1979) Kinetics and equilibria of pyrimidine nucleoside monophosphate kinase from human erythrocytes. *Biochim Biophys Acta* **571:**45–54.
Serina L, Blondin C, Krin E, Sismeiro O, Danchin A, Sakamoto H, Gilles AM, and Barzu O (1995) *Escherichia coli* UMP-kinase, a member of the aspartokinase family, is a hexamer regulated by guanine nucleotides and UTP. *Biochemistry* **34:**5066–5074.
Teng YS, Chen SH, and Scott R (1976) Human erythrocyte pyrimidine nucleoside monophosphate kinase. Partial purification and properties of two allelic gene products. *J Biol Chem* **251:**4179–4183.
Townsley CA, Chi K, Ernst DS, Belanger K, Tannock I, Bjarnason GA, Stewart D, Goel R, Ruether JD, Siu LL, et al. (2003) Phase II study of troxacitabine (BCH-4556) in patients with advanced and/or metastatic renal cell carcinoma: a trial of the National Cancer Institute of Canada-Clinical Trials Group. *J Clin Oncol* **21:**1524–1529.
Traut TW (1994a) Dissociation of enzyme oligomers: a mechanism for allosteric regulation. *Crit Rev Biochem Mol Biol* **29:**125–163.
Traut TW (1994b) Physiological concentrations of purines and pyrimidines. *Mol Cell Biochem* **140:**1–22.
Van Rompay AR, Johansson M, and Karlsson A (1999) Phosphorylation of deoxycytidine analog monophosphates by UMP-CMP kinase: molecular characterization of the human enzyme. *Mol Pharmacol* **56:**562–569.
Van Rompay AR, Johansson M, and Karlsson A (2000) Phosphorylation of nucleosides and nucleoside analogs by mammalian nucleoside monophosphate kinases. *Pharmacol Ther* **87:**189–198.
Weitman S, Marty J, Jolivet J, Locas C, and Von Hoff DD (2000) The new dioxolane, (−)-2′-deoxy-3′-oxacytidine (BCH-4556, troxacitabine), has activity against pancreatic human tumor xenografts. *Clin Cancer Res* **6:**1574–1578.
Wiesmuller L, Scheffzek K, Kliche W, Goody RS, Wittinghofer A, and Reinstein J (1995) Crystallization and preliminary X-ray analysis of UMP/CMP-kinase from *Dictyostelium discoideum* with the specific bisubstrate inhibitor P1-(adenosine 5′)-P5-(uridine 5′)-pentaphosphate (UP5A). *FEBS Lett* **363:**22–24.
Yan H and Tsai MD (1999) Nucleoside monophosphate kinases: structure, mechanism and substrate specificity. *Adv Enzymol Relat Areas Mol Biol* **73:**103–134.
Yu L, Mack J, Hajduk PJ, Kakavas SJ, Saiki AY, Lerner CG, and Olejniczak ET (2003) Solution structure and function of an essential CMP kinase of *Streptococcus pneumoniae*. *Protein Sci* **12:**2613–2621.

Address correspondence to: Dr. Yung-Chi Cheng, Department of Pharmacology, Yale University School of Medicine, 333 Cedar St., SHM B226, New Haven, CT 06520. E-mail: yccheng@yale.edu