# Cloning of Deoxynucleoside Monophosphate Kinase Genes and Biosynthesis of Deoxynucleoside Diphosphates

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**Abstract:** The genes encoding four deoxynucleoside monophosphate kinase (dNMP kinase) enzymes, including *ADK1* for deoxyadenylate monophosphate kinase (AK), *GUK1* for deoxyguanylate monophosphate kinase (GK), *URA6* for deoxycytidylate monophosphate kinase (CK), and *CDC8* for deoxythymidylate monophosphate kinase (TK), were isolated from the genome of *Saccharomyces cerevisiae* ATCC 2610 strain and cloned into *E. coli* strain BL21(DE3). Four recombinant plasmids, pET17b-JB1 containing *ADK1*, pET17b-JB2 containing *GUK1*, pET17b-JB3 containing *URA6*, and pET17b-JB4 containing *CDC8*, were constructed and transformed into *E. coli* strain for overexpression of AK, GK, CK, and TK. The amino acid sequences of these enzymes were analyzed and a putative conserved peptide sequence for the ATP active site was proposed. The four deoxynucleoside diphosphates (dNDP) including deoxyadenosine diphosphate (dADP), deoxyguanosine diphosphate (dGDP), deoxycytidine diphosphate (dCDP), and deoxythymidine diphosphate (dTDP), were synthesized from the corresponding deoxynucleoside monophosphates (dNMP) using the purified AK, GK, CK, and TK, respectively. The effects of pH and magnesium ion concentration on the dNDP biosynthesis were found to be important. A kinetic model for the synthetic reactions of dNDP was developed based on the Bi–Bi random rapid equilibrium mechanism. The kinetic parameters including the maximum reaction velocity and Michaelis–Menten constants were experimentally determined. The study on dNDP biosynthesis reported in this article are important to the proposed bioprocess for production of deoxynucleoside triphosphates (dNTP) that are used as precursors for in vitro DNA synthesis. There is a significant advantage of using enzymatic biosyntheses of dNDP as compared to the chemical method that has been in commercial use. 

**Keywords:** deoxynucleoside monophosphate kinases (dNMP kinases); dNMP kinase genes; deoxynucleoside diphosphate (dNDP) biosynthesis; deoxynucleoside monophosphate (dNMP); *Saccharomyces cerevisiae* genomic DNA; expression in *E. coli*

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## INTRODUCTION

Deoxynucleoside triphosphates (dNTP) comprise four components, deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and deoxythymidine triphosphate (dTTP). Polymerization reactions involved in DNA biosynthesis and transcription of DNA require dNTP as precursor subunits. Due to the great demand for DNA biosynthesis and polymerase chain reaction (PCR) from the research community and biotechnology industries, it is clear that the demand for dNTP will continue to increase (Erlich et al., 1991; Pavlov et al., 2004). Currently, dNTP is commercially produced by chemical method (Chambers and Khorana, 1957; Chambers et al., 1957; Smith and Khorana, 1958). This method involves the reaction of tri-*n*-butylammonium salts of deoxynucleoside monophosphates (dNMP) and orthophosphoric acid with dicyclohexylcarbodiimide (DCC) in the aqueous pyridine or dimethylformamide (DME). The chemical method gives relatively low yield of dNTP, and pyridine and DMF are toxic as listed in the toxic substance list of Environmental Protection Agency (EPA, 2001).

In view of economic consideration and environmental problem related to the chemical method, we proposed an enzymatic method as an alternative for production of dNTP (Bao and Ryu, 2005; Bao et al., 2005). The dNTP biosynthesis requires two-step enzymatic phosphorylation reactions starting from dNMP to deoxynucleoside diphosphates (dNDP) and to dNTP in the second step. Four different deoxynucleoside monophosphate kinases (dNMP kinases) are required for the first-step phosphorylation reaction, dNMP to dNDP. No toxic solvent is used in the proposed bioprocess. A complete bioconversion from dNMP to dNTP can be obtained, therefore the dNTP purification is significantly simplified and the production cost can also be reduced significantly (Bao and Ryu, 2005). The four enzymes required for the enzymatic method include: deoxyadenylate monophosphate kinase (AK, EC 2.7.4.3) for phosphorylation

of deoxyadenosine monophosphate (dAMP) to deoxyadenosine diphosphate (dADP), deoxyguanylate monophosphate kinase (GK, EC 2.7.4.8) for deoxyguanosine monophosphate (dGMP) to deoxyguanosine diphosphate (dGDP), deoxycytidylate monophosphate kinase (CK, EC 2.7.4.14) for deoxycytidine monophosphate (dCMP) to deoxycytidine diphosphate (dGDP), and deoxythymidylate monophosphate kinase (TK, EC 2.7.4.9) for deoxythymidine monophosphate (dTMP) to deoxythymidine diphosphate (dTDP).

In this study, the genes encoding these four enzymes from *Saccharomyces cerevisiae* strain were selected because the specific activity of gene products are significantly higher compared to those from other organisms. Except for GK (Li et al., 1996), the cloning of the other three yeast kinase genes and the biosynthesis of dNDP from dNMP have not been studied in detail or reported. Our goals were to study the cloning and expression of the four yeast dNMP kinases in *E. coli* and the reaction mechanism and kinetics of the phosphorylation reaction of dNMP to dNDP. The outcome of this study will enable us to design the proposed bioprocess producing dNTP from dNMP. The bioprocess producing dNTP from dNDP has been reported earlier (Bao and Ryu, 2005; Bao et al., 2005). More specific goals are to: (1) clone the genes encoding the four enzymes from *S. cerevisiae* into *E. coli*, (2) investigate the expression of the genes in *E. coli*, (3) investigate the effects of pH and $Mg^{2+}$concentration on the initial reaction rate and determine the optimal pH and $Mg^{2+}$concentration for the enzyme reactions, and (4) develop the kinetic model based on the proposed Bi–Bi random rapid equilibrium mechanism and determine the kinetic parameters for these enzyme reactions.

## MATERIALS AND METHODS

### Materials

All chemicals and enzymes used in this study, including 2′-deoxyadenosine 5′-monophosphate sodium salt (dAMP), 2′-deoxyguanosine 5′-monophosphate sodium salt (dGMP), 2′-deoxycytidine 5′-monophosphate sodium salt (dCMP), 2′-deoxythymidine 5′-monophosphate sodium salt (dTMP), phosphoenolpyruvic acid monopotassium salt (PEP), pyruvate kinase from rabbit muscle (PK), L-lactate dehydrogenase (LDH) from porcine heart (LDH), lyticase, nicotinamide adenine dinucleotide (NADH-reduced form), and isopropyl-beta-D-thiogalactopyranoside (IPTG) were purchased from Sigma-Aldrich (St. Louis, MO). Lysozyme is from Roche (Indianapolis, IN). Tween 20 and Nonidet P40 were from MP Biomedicals (Irvine, CA). The LB medium used for *E. coli* culture contains 10 g peptone, 5 g yeast extract, and 5 g sodium chloride per liter deionized $H_2O$ with 100 μg/mL of ampicillin. The YPD medium used for *S. cerevisiae* culture contains 10 g yeast extract, 20 g peptone, 20 g glucose per liter of deionized $H_2O$. Twenty grams agar was added per liter of LB or YPD medium solution to make the plates for solid culture.

### Organisms and Vectors

*S. cerevisiae* ATCC 2601 NRRL Y-53 strain was purchased from ATCC (Manassas, VA). The cloning vector plasmids pUC19 and pGEM-T-easy and its host *E. coli* strain DH5alpha, the expression vector pET17b and its host *E. coli* strain BL21(DE3) were purchased from Novagen (Madison, WI).

### Gene Sources

The genomic DNA of *S. cerevisiae* ATCC 2601 NRRL Y-53 was prepared using Qiagen DNeasy Tissue Kit (Valencia, CA). The frozen and dried yeast cells was diluted in deionized $H_2O$ and streaked onto a YPD plate. The cells were grown for 4 days at ambient temperature. A colony was picked up and transferred to 5 mL YPD medium and grew overnight at 30°C. The yeast cell wall was lysed with lyticase and the genomic DNA was purified using the Qiagen DNeasy Tissue Kit and following the Yeast Genomic DNA Purification Protocol (Valencia, CA). The concentration of purified genomic DNA obtained was measured using UV spectrophotometer at 260 nm. Fifty micrograms per milliliter of DNA has an absorbance of 1.0 at 260 nm.

### Amplification of Genes and Cloning

*ADK1*, *GUK1*, *URA6*, and *CDC8* were amplified by PCR reaction using the genomic DNA of *S. cerevisiae* as template. The gene sequences of *ADK1*, *GUK1*, *URA6*, and *CDC8* were obtained from *Saccharomyces* Genome Database (http://www.yeastgenome.org/). The oligonucleotide primers were designed using the Web Primer tool (http://seq.yeastgenome.org/cgi-bin/web-primer) and synthesized by Operon Technologies (Huntsville, AL). The PCR primers used are: for *ADK1*, 5′-CGGGATCCCATATGTCTAGCTCAGAATCCAT-3′ (forward with *Nde*I and *Bam*HI) and 5′-GGGCCGGAATTCTTAATCCTTACCTAGCTTGTT-3′ (reverse with *Eco*RI); for *GUK1*, 5′-GCTCTAGAATTAATATGTCCCGTCCTATCGTAAT-3′ (forward with *Ase*I and *Xba*I) and 5′-CGGGATCCTCATTTTTCTGCAAAGATAA-3′ (reverse with *Bam*HI); for *URA6*, 5′-CGGGATCCCATATGACAGCTGCCACTACATCAC-3′ (forward with *Nde*I and *Bam*HI) and 5′-GGGCCGGAATTCCTATAAGCTATCACGGATAGCGTGTT-3′ (reverse with *Eco*RI); and for *CDC8*, 5′-GGAATTCCATATGATGATGGGTCGTGGCAAATT-3′ (forward with *Nde*I) and 5′-CGGAATTCCTAGAAGAACGAAAATTTATCA-3′ (reverse with *Eco*RI).

The pUC19 (or pGEM-T-easy) and pET17b plasmids were used as the cloning and expression vectors, respectively. The PCR product was cloned into pUC19 or pGEM-T-easy vector and then transformed into *E. coli* strain DH5alpha. The kinase genes contained in the recombinant with pUC19 or pGEM-T-easy plasmids was sequenced using the corresponding PCR primers for the confirmation of the gene sequence. Then the kinase gene fragments were cleaved, gel purified, and ligated into pET17b expression vector and transformed into the host *E. coli* strain BL21(DE3) for

over-expression of dNMP kinases. The T7 tag in pET17b was deleted and the translation of the kinase genes without T7 tag gave the kinases without fusion proteins.

### Expression of Kinase in *E. coli*

One liter fermentation medium was inoculated with 0.5 mL of overnight culture of freshly transformed *E. coli* strain BL21(DE3) containing expression plasmids in LB medium with 100 μg/mL of ampicillin and cultured for about 12 h till it reached $OD_{600} = 0.6$. The culture temperature was 37°C for AK, GK, TK expression, and 25°C for CK expression, respectively. Cells were harvested and washed with Buffer A (50 mM Tris-HCl, 50 mM dextrose, and 1 mM EDTA, pH 7.9).

The cells were resuspended in 50 mL of pre-lysis buffer (10 mM Tris-HCl, 50 mM KCl, 1 mM EDTA, 4 mg/mL lysozyme, pH 7.9) and incubated for 30 min at ambient temperature. Fifty milliliters of lysis Buffer (10 mM Tris-HCl, 50 mM KCl, 1 mM EDTA, 1 mM PMSF, 0.5% Tween 20, 0.5% Nonidet P40, pH 7.9) was added and the cells were incubated for 30 min at room temperature and another 30 min at 37°C. The lysate mixture was centrifuged and 60 g of powdered ammonium sulfate was added to the supernatant obtained from the 1 L whole broth. The protein precipitate was recovered by centrifugation in 20 mL of Buffer A. The enzyme was eluted with Buffer A from solution through a Sephadex G-100 column ($80 \times 1$ cm) with Buffer A, monitoring protein by a UV detector at 280 nm and SDS–PAGE. All the SDS–PAGE protein samples were analyzed on 15% polyacylamide gel and stained with Coomassie brilliant blue R-250.

The enzyme activity of the collected fractions was assayed using spectrophotometer at 354 nm. The assay was carried out at 30°C in a 0.5 mL quartz cuvette containing 50 mM Tris buffer solution (pH 8.0), 100 mM KCl, 4 mM $MgCl_2$, 10 U PK per mL, 10 U LDH per mL, 10 mg NADH per mL, 0.5 mM PEP, 1.0 mM dNMP, and 1.0 mM ATP.

Fifty parts of Reagent A (2% sodium carbonate, 1% biocinchoninic acid, 0.16% sodium tartrate, 0.4% sodium hydroxide, and 0.95% sodium bicarbonate) and one part of Reagent B (4% $CuSO_4$) were mixed to make the working solution for the protein assay. The assay tubes were incubated at 60°C for 30 min. The tubes were cooled to ambient temperature and the absorbance was read at 562 nm in 1 mL plastic cuvette. The protein concentration was calibrated using BSA.

### Determination of Kinetic Parameters

The initial reaction rates were measured in a 1 mL quartz cuvette containing 0.5 mL of 50 mM Tris buffer solution and 100 mM potassium chloride. The pH of the buffer was adjusted to 8.0. The progress of reaction was monitored by using Beckman DU-640 spectrophotometer. Each reaction cell contained 10 μL of NADH (10 mg/mL), 10 μL of LDH solution (500 U/mL), 10 μL of pyruvate kinase solution (500 U/mL), and 25 μL PEP solution (10 mM), all in 0.5 mL 50 mM Tris buffer solution (pH 8.0). Each reaction cell contained varying concentrations of dNMP, ATP, and $Mg^{2+}$. The reaction temperature was maintained at 30°C.

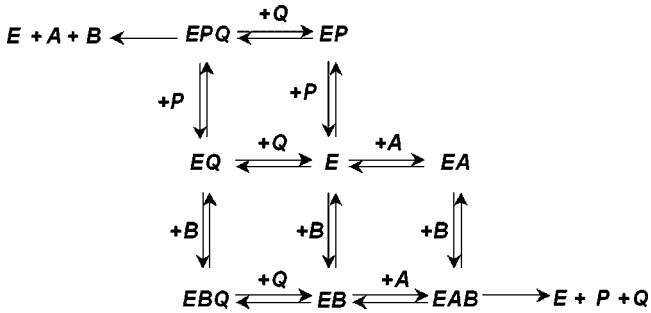



**Figure 1.** Proposed reaction mechanism of Bi–Bi random rapid equilibrium.

### Kinetic Model and Reaction Mechanism

The reaction mechanism proposed here based on the Bi–Bi random rapid equilibrium model for biosyntheses of dNDP by dNMP kinases and shown in Figure 1 was adapted (Leskovac, 2003). In this reaction mechanism, the reaction is reversible and only the forward reaction for dNDP formation was investigated. dNMP (*B*) reacts only with ATP after ATP forms the bivalent metal–ion complex MgATP (*A*) to produce the products MgADP (*Q*) and dNDP (*P*), as shown in Reaction Equation (1) (see "Nomenclature" for the symbols).

$$\text{MgATP } (A) + \text{dNMP } (B) \rightleftharpoons \text{MgADP } (Q) + \text{dNDP } (P) \quad (1)$$

As shown in Figure 1, MgATP (*A*) and MgADP (*Q*), dNMP (*B*) and dNDP (*P*) compete for the ATP site and dNMP site of the enzymes (*E*), respectively. dNDP (*P*) is a competitive inhibitor of MgATP (*A*) because of the possible partial-overlap of the two active sites. MgADP (*Q*) is a non-competitive inhibitor of dNMP (*B*) because MgADP (*Q*) forms an inactive ternary complex with enzyme (*E*) and Mg-dNMP (*B*). The expression for the initial forward reaction rate neglecting the formation of products ([*P*] and $[Q] = 0$) was obtained from the rapid equilibrium models as Equation (2):

$$\frac{v}{V_{\max}} = \frac{11}{11 + K_{M,A}/[A] + K_{M,B}/[B] + K_{M,A}K_{M,AB}/[A][B]} \quad (2)$$

Equation (2) is used as the working equation for determination of kinetic parameters.

## RESULTS

### Cloning and Over-Expression of dNMP Kinase Genes

Important information on cloning and over-expression of the dNMP kinase genes is summarized in Table I. The results of

**Table I.** Cloning and over-expression of deoxynucleoside monophosphate kinase (dNMP kinases).

| dNMP kinase gene | *ADK1* | *GUK1* | *URA6* | *CDC8* |
|---|---|---|---|---|
| Gene name and in SGD[a] | YDR226W | YDR454C | YKL024C | YJR057W |
| Primary SGDID[b] | S000002634 | S000002862 | S000001507 | S000003818 |
| Gene length (bp) | 669 | 564 | 615 | 651 |
| Protein MW (Da) | 24,255 | 20,637 | 22,933 | 24,687 |
| Cloning plasmid | PUC19-*ADK1* | pUC19-*GUK1* | pUC19-*URA6* | pUC19-*CDC8* |
| Expression plasmid | PET17b-JB1 | pET17b-JB2 | pET17b-JB3 | pET17b-JB4 |
| Effect of IPTG inducer | No | No | No | No |
| Cultivation temperature[c] | 37°C | 37°C | 25°C | 37°C |
| Crude enzyme productivity (g/L) | 0.63 | 0.56 | 0.90 | 0.32 |
| Specific activity (μmol/min mg protein)[d] | 100 | 20 | 80 | 30 |

The gene source is from *Saccharomyces cerevisiae* ATCC 2601 strain. The host organism is *E. co*li strain BL21(DE3).
[a]SGD: *Saccharomyces* Genome Database (http://www.yeastgenome.org/).
[b]SGDID: the identifying number of a specific gene within SGD.
[c]Cultivation temperature at which formation of inclusion body was prevented.
[d]Crude enzyme specific activity.

SDS–PAGE gel electrophoresis of the kinase expression under different IPTG induction conditions are shown in Figure 2.

AK is a commercially available enzyme generally obtained from animal tissues (Callaghan, 1957; Noda, 1958, 1973; Kuby et al., 1978; Ladner and Whitesides, 1985). *ADK1* was selected as the target gene from the AK gene family of *S. cerevisiae*, *ADK1*, *ADK2*, and *ADK3*, because of relatively high activity of its gene product. The PCR product was cloned into the *Bam*HI–*Eco*RI sites of pUC19 to give the pUC19-*ADK1* plasmid and then transformed into *E. coli* strain DH5alpha for amplification. The pUC19-*ADK1* plasmid was sequenced and no mutation inside the *ADK1* gene was found. The *Nde*I/*Eco*RI fragment of pUC19/*ADK1* plasmid was cleaved, gel purified, and ligated into pET17b to give the expression plasmid designated as pET17b-JB1. Then pET17b-JB1 was transformed into the host *E. coli* strain BL21(DE3) for over-expression of AK enzyme. Figure 2a shows that a high expression level of AK is obtained and there is almost no difference in AK expression level with and without IPTG induction.

*GUK1* encoding GK from *S. cerevisiae* has been cloned into *E. coli* in laboratory scale (Konrad, 1992; Li et al., 1996). Li et al. (1996) in their construction of the experiment vector for *GUK1*, used *Nde*I restriction enzyme resulting in the gene fragment containing only the partial *GUK1*. When the same approach was tested it was found that the construction of *GUK1* expression plasmid was unsuccessful. There is one *Nde*I restriction site contained in the *GUK1*. In this study, we constructed a new plasmid for the yeast GK expression. The PCR product was cloned into the *Xba*I–*Bam*HI sites of pUC19 to give the pUC19-*GUK1* plasmid and transformed into *E. coli* strain DH5alpha. The pUC19-*GUK1* was sequenced and no mutation inside *GUK1* was found. An *Ase*I site in the forward primer was selected for *GUK1* gene. The *Ase*I/*Bam*HI fragment of pUC19-*GUK1* plasmid was introduced into *Nde*I/*Bam*HI sites of pET17b because the two base pairs overhang sequence (TA) of *Ase*I and *Nde*I restriction sites are the same for ligation. This cloning vector was transformed into *E. coli* strains BL21(DE3). The resulting expression plasmid was designated as pET17b-JB2. Figure 2b shows that a high expression level of GK was obtained, and no significant effect of IPTG on GK expression was found.

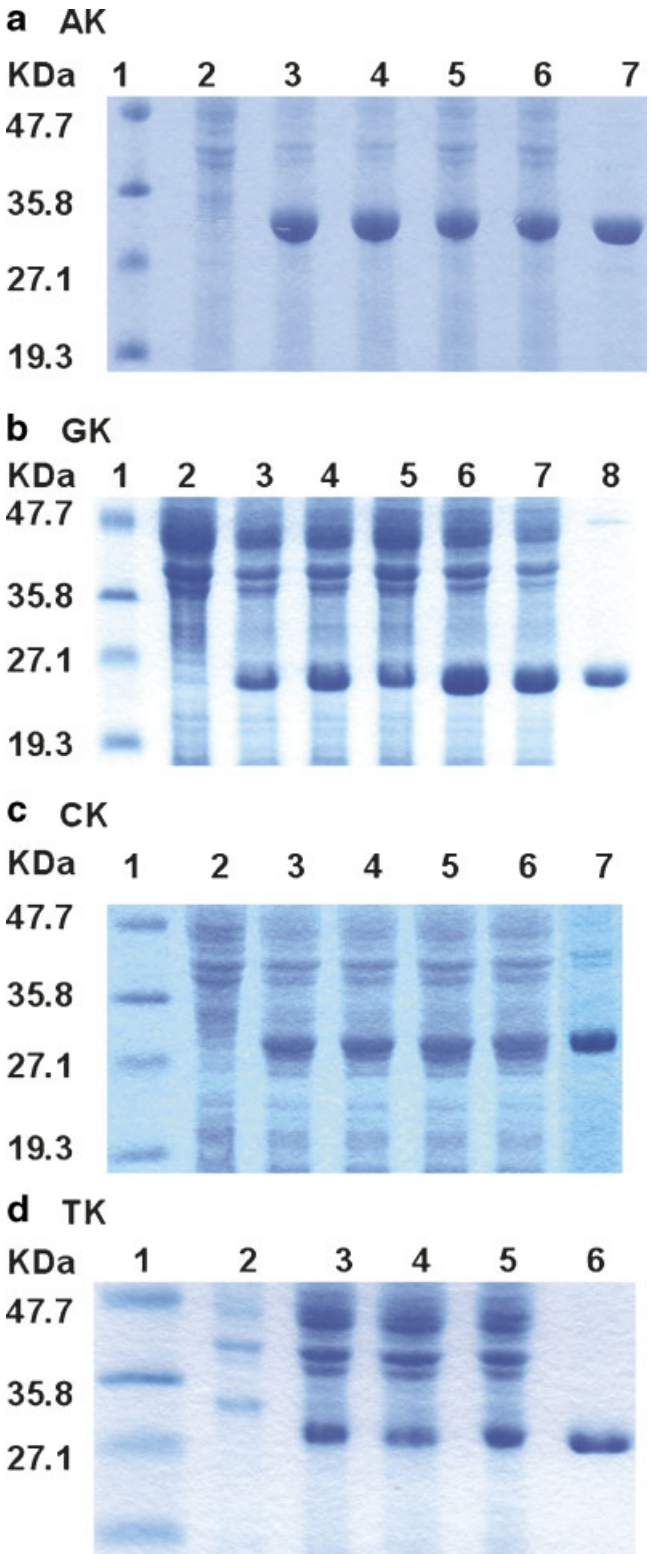
*URA6* encoding both uridine monophosphate kinase and deoxycytidylate monophosphate kinase from *S. cerevisiae* has been purified and characterized from the wild type *S. cerevisiae* strain (Jong et al., 1993; Liljelund and Lacroute, 1986; Ma et al., 1990). The PCR product was cloned into the *Bam*HI–*Eco*RI sites of pUC19 to give the pUC19-*URA6* cloning plasmid and then transformed into *E. coli* strain DH5alpha. *URA6* was sequenced and no mutation was found. The *Nde*I/*Bam*HI fragment of pUC19-*GUK1* plasmid was introduced into *Nde*I/*Eco*RI digested pET17b to give the expression plasmid designated as pET17b-JB3, and then transformed into *E. coli* strain BL21(DE3). Since the CK expression at 37°C formed insoluble inclusion body, the expression of CK was carried out at 25°C to get the soluble protein. Figure 2c indicates the high expression level of CK and the expression level of CK was found independent from IPTG.

*CDC8* from *S. cerevisiae* has been purified and characterized from the wild type *S. cerevisiae* strain (Kohno et al., 1983; Jong and Campbell, 1984; Jong et al., 1984). The PCR product was cloned into pGEM-T-easy vector to give the pGEM-T/*CDC8* plasmid and then transformed into *E. coli* strain DH5alpha. *CDC8* was sequenced and no mutation was found. The *Nde*I/*Eco*RI fragment of pGEM-T-*CDC8* plasmid was introduced into *Nde*I/*Eco*RI digested pET17b to give the expression plasmid designated as pET17b-JB4, and transformed into the *E. coli* strain BL21(DE3). Similar to three other dNMP kinases, Figure 2d shows a high expression level and independence of expression level from IPTG.

## Analysis of Amino Acid Sequence of the dNMP Kinases

The amino acid sequence of the four dNMP kinases cloned and expressed were deduced by translating the gene sequence and analyzed using Vector NTI 9.0 (InforMax, Bethesda, MD, and Invitrogen, Carlsbad, CA). The similarity table of the amino acid alignment shown in Figure 3 revealed only

approximately 20% homology similarity among the four dNMP kinase enzymes. However, a highly conserved consensus sequence, GxxxxGKxTxxxxL (GGKTL), was found with high similarity in the region near to N-terminal of the peptides in all four dNMP kinases. The conserved GGKTL sequence of the four dNMP enzymes may contain the sequence of the NTP site of the dNMP enzymes, which orients and directs the universal phosphate donors such as ATP into this sites (Yan and Tsai, 1999).

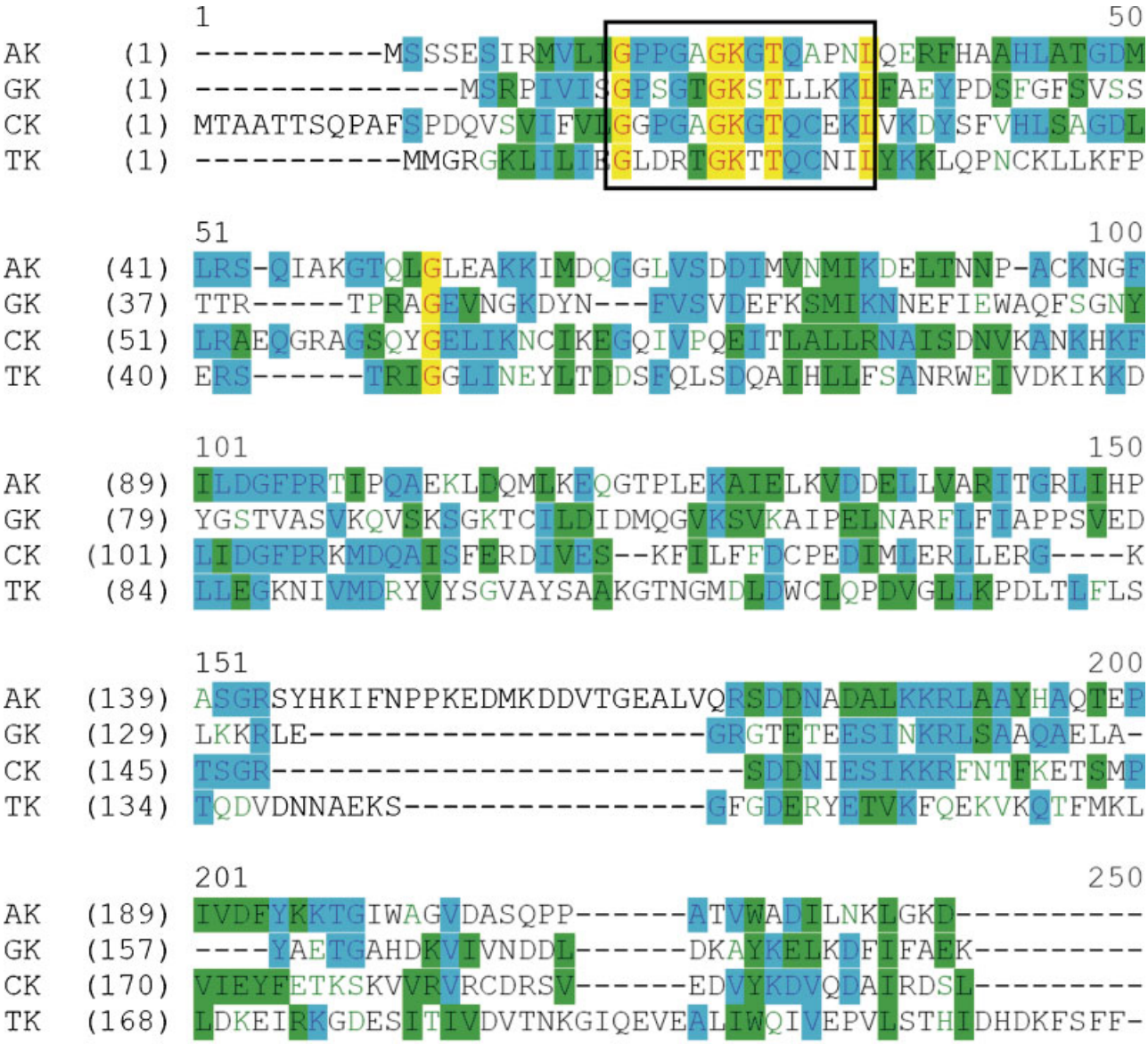



## Effects of pH and $Mg^{2+}$ Concentration on Initial Reaction Rates

Figure 4 shows very similar pH dependence of the reaction rate profiles for the four dNMP kinase enzyme reactions. The initial reaction rates for all four dNDP syntheses catalyzed by dNMP kinases increase with increasing pH at two different $Mg^{2+}$ levels. The reaction rates reach maxima at about pH 8 for the syntheses of dADP, dGDP, and dCDP then decline with further increasing pH. For dTDP synthesis, the reaction rate reaches maximum at pH $7 \sim 8$ then decline with further increasing pH. The results are similar to that of the enzyme reactions using the same enzyme but different substrates, nucleoside monophosphates (NMPs) such as AMP, GMP, CMP, and TMP (Jong and Campbell, 1984; Konrad, 1992, 1993; Liljelund and Lacroute, 1986; Ma et al., 1990). The optimal pH for the dNDP synthesis coincides with that of the pyruvate kinase catalyzed phosphorylation from dNDP to dNTP, the second step phosphorylation in the dNTP biosynthesis from dNMP (Bao and Ryu, 2005; Bao et al., 2005).

All four dNMP kinases require $Mg^{2+}$ as activator by orienting and directing ATP and dNMP to the corresponding active sites (Noda, 1973; Yan and Tsai, 1999). Figure 5 indicates that the initial reaction rates for all four dNDP syntheses increase with increasing $Mg^{2+}$ concentration at varying substrate concentrations. The reaction rates reach maxima then remain practically constant. The results show that the $Mg^{2+}$ requirement in all four phosphorylation reactions was affected by not only ATP concentration but also the dNMP concentration. The reaction rates reach the maxima at the ratio of $[Mg^{2+}]/([ATP]+[dNMP])$ equals 2 for dADP, dGDP, and dCDP syntheses, and 2.5 for dTDP synthesis. Therefore, the saturation concentration of $Mg^{2+}$ was determined as in the ratio of $[Mg^{2+}]/([ATP]+[dNMP])$ equals 2 for dADP, dGDP, and dCDP syntheses, and 2.5 for dTDP synthesis.

**Figure 2.** Expression of deoxynucleoside monophosphate kinases (dNMP kinases) (deoxyadenylate monophosphate kinase (AK), deoxyguanylate monophosphate kinase (GK), deoxycytidylate monophosphate kinase (CK), and deoxythymidylate monophosphate kinase (TK)) under different IPTG induction. Panel (**a**) AK expression in BL21(DE3) cells containing pET17b-JB1 at 37°C. **Lane 1**, protein MW markers; **lane 2**, pET17b control with no IPTG; **lane 3**, no IPTG; **lane 4**, 0.5 mM IPTG; **lane 5**, 1.0 mM IPTG; **lane 6**, 2.0 mM IPTG; **lane 7**, purified AK. Panel (**b**) GK expression in BL21(DE3) cells containing pET17b-JB2 at 37°C. **Lane 1**, protein MW markers; **lane 2**, pET17b control with no IPTG; **lane 3**, no IPTG; **lane 4**, 0.5 mM IPTG; **lane 5**, 1.0 mM IPTG; **lane 6**, 2.0 mM IPTG; **lane 7**, 3.0 mM IPTG; **lane 8**, purified GK. Panel (**c**) CK expression in BL21(DE3) cells containing pET17b-JB3 at 25°C. **Lane 1**, protein MW markers; **lane 2**, pET17b control with no IPTG; **lane 3**, no IPTG; **lane 4**, 0.5 mM IPTG; **lane 5**, 1.0 mM IPTG; **lane 6**, 2.0 mM IPTG; **lane 7**, purified CK. Panel (**d**) TK expression in BL21(DE3) cells containing pET17b-JB4 at 37°C. **Lane 1**, protein MW markers; **lane 2**, pET17b control with no IPTG; **lane 3**, no IPTG; **lane 4**, 0.5 mM IPTG; **lane 5**, 1.0 mM IPTG; **lane 6**, purified TK. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]




Similarity table of the dNMP enzymes (%)

| | AK | GK | CK | TK |
|---|---|---|---|---|
| AK | 100 | 22 | 28 | 13 |
| GK | | 100 | 25 | 21 |
| CK | | | 100 | 18 |
| TK | | | | 100 |

**Figure 3.** Amino acid sequence alignment of AK, GK, CK, and TK translated from the gene sequences. The consensus residues GxxxxGKxTxxxxL (GGKTL) are boxed. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

## Kinetic Parameters

The kinetic parameters including the maximum reaction velocity and Michaelis–Menten constants were determined at 30°C, pH 8.0, and the saturation concentration of $Mg^{2+}$ for dNDP synthesis using the four dNMP kinases. The results are summarized in Table II. The working equations for Michaelis constant determination derived from Equation (2) are given in Equation (3) for the [*B*] (MgdNMP) = constant. For the initial reaction rate determination, the concentration of products [*P*] (dNDP) and [*Q*] (MgADP) are assumed to be zero ([*P*] = 0 and [*Q*] = 0).

$$\frac{1}{v} = \frac{K_{M,AB}K_{M,A}}{V_{max}}\left(\frac{1}{[B]} + \frac{1}{K_{M,B}}\right)\frac{1}{[A]} + \frac{1}{V_{max}}\left(\frac{K_{M,AB}}{[B]} + 1\right) \quad (3)$$

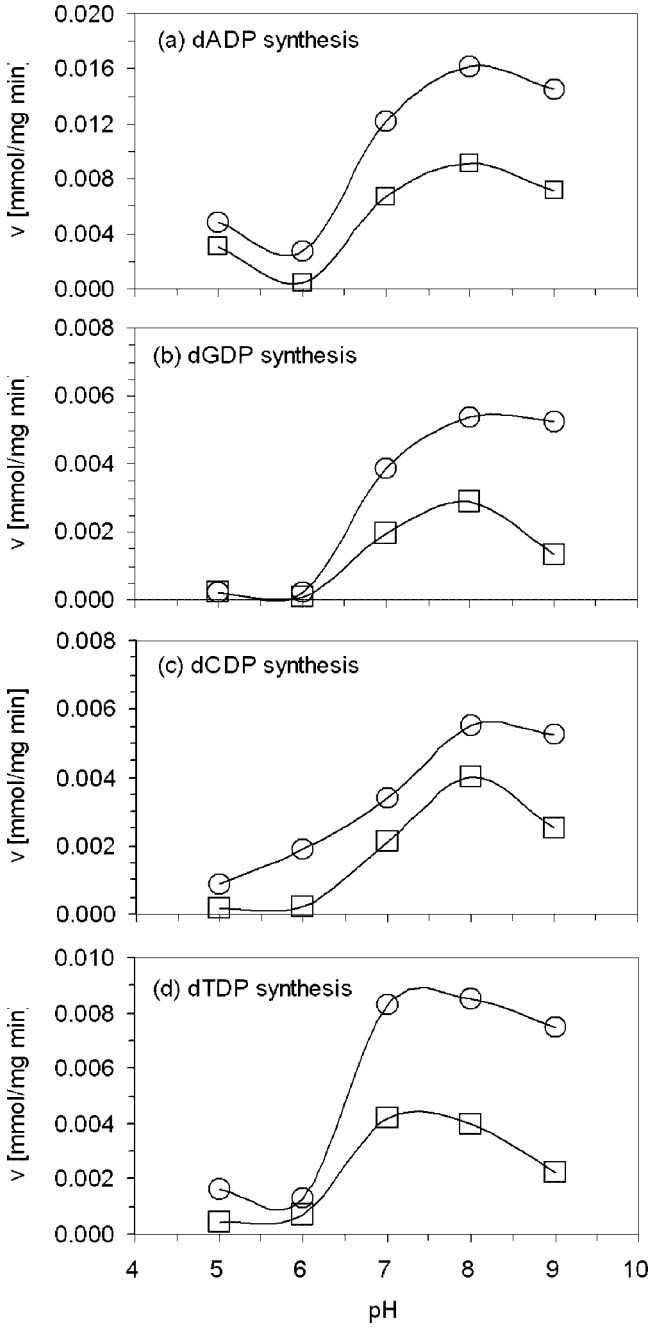



**Figure 4.** Effect of pH on the initial reaction rates at varying $Mg^{2+}$ concentrations. Reaction conditions: 50 mM Tris Cl, 30°C, $K^+$ 100 mM, PK 10 U/mL, L-lactate dehydrogenase (LDH) 10 U/mL, PEP 0.5 mM, ATP 1.0 mM, deoxynucleoside monophosphates (dNMP) 1.0 mM. pH 5 ~ 9. ○, $Mg^{2+}$ 1.5 mM; □, $Mg^{2+}$ 1.0 mM. **a**: Deoxyadenosine diphosphate (dADP) synthesis; (**b**) deoxyguanosine diphosphate (dGDP) synthesis; (**c**) deoxycytidine diphosphate (dCDP) synthesis; and (**d**) deoxythymidine diphosphate (dTDP) synthesis.

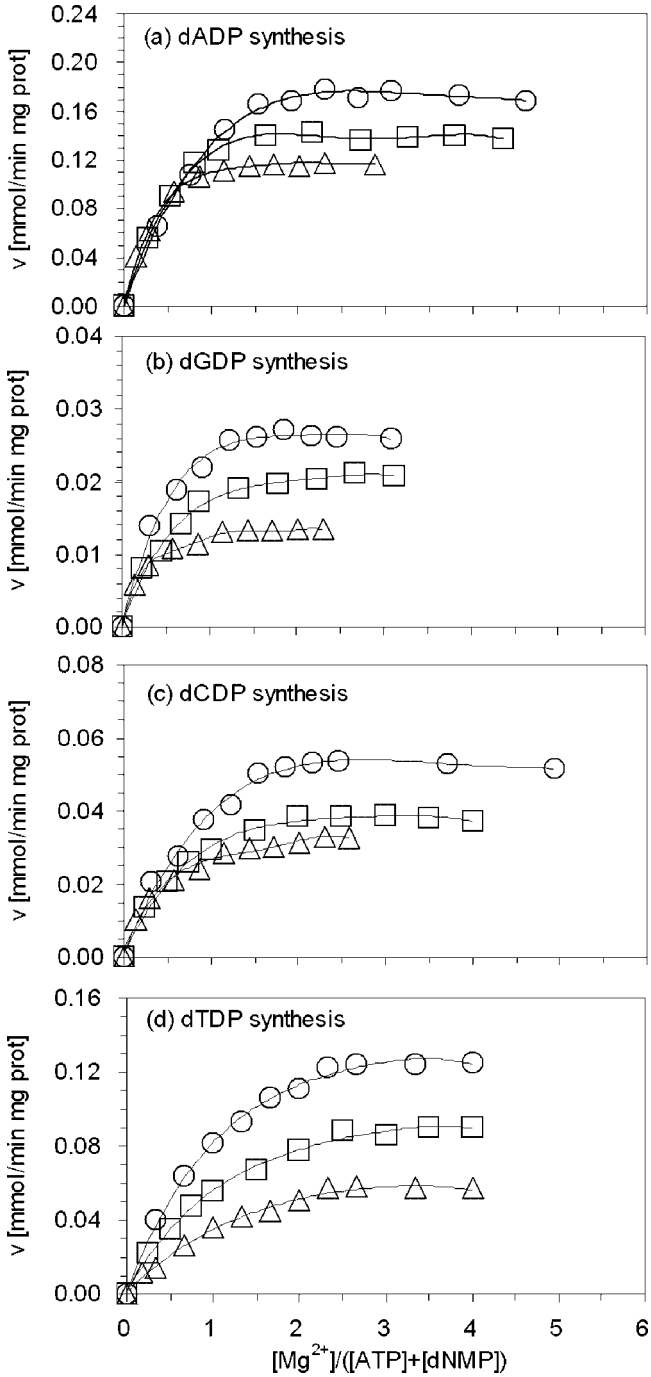



**Figure 5.** Effect of $Mg^{2+}$ concentration on the initial reaction rates at varying substrate concentrations. Reaction conditions: 50 mM Tris Cl (pH 8.0), 30°C, $K^+$ 100 mM, PK 10 U/mL, LDH 10 U/mL, PEP 0.5 mM, dNMP 1.0 mM, $Mg^{2+}$ 0–8 mM. ○, ATP 0.37 mM; □, ATP 0.62 mM; △, ATP 1.12 mM. **a**: dADP synthesis; (**b**) dGDP synthesis; (**c**) dCDP synthesis; and (**d**) dTDP synthesis.

## DISCUSSION

The proposed dNTP biosynthesis process requires two-step enzymatic phosphorylation reactions starting from the dNMP to dNDP and to the dNTP in the second step. In the first-step phosphorylation reactions from dNMP to dNDP, the reactions are catalyzed by four different dNMP kinases, AK, GK, CK, and TK. Among the four enzymes required, only AK obtained from animal tissues is commercially available to date (Callaghan, 1957; Kuby et al., 1978; Noda, 1958, 1973; Sigma-Aldrich Biochemical and Reagents, 2004/2005). The other three enzymes, GK, CK, and TK are

**Table II.** Kinetic parameters of deoxynucleoside diphosphates (dNDP) syntheses catalyzed by dNMP kinase enzymes.

| Parameters | Deoxyadenosine diphosphate (dADP) synthesis | Deoxyguanosine diphosphate (dGDP) synthesis | Deoxycytidine diphosphate (dCDP) synthesis | Deoxythymidine diphosphate (dTDP) synthesis |
|---|---|---|---|---|
| $V_{max}$ (μmol/min mg protein)[a] | $220 \pm 13.2$ | $64 \pm 3.8$ | $120 \pm 10.8$ | $430 \pm 25.6$ |
| $K_{M,A}$ (mM) | $0.28 \pm 0.03$ | $0.67 \pm 0.04$ | $0.11 \pm 0.02$ | $0.48 \pm 0.10$ |
| $K_{M,B}$ (mM) | $1.10 \pm 0.1$ | $0.33 \pm 0.02$ | $0.53 \pm 0.08$ | $0.89 \pm 0.18$ |
| $K_{M,BA}$ (mM) | $0.18 \pm 0.02$ | $0.50 \pm 0.04$ | $0.062 \pm 0.01$ | $0.27 \pm 0.05$ |
| $K_{M,AB}$ (mM) | $0.71 \pm 0.07$ | $0.25 \pm 0.02$ | $0.30 \pm 0.05$ | $0.50 \pm 0.10$ |

Reaction conditions used: 30°C, pH 8.0, and saturated concentrations of $Mg^{2+}$ and $K^{+}$.
[a] $V_{max}$ for purified enzymes.

not commercially available. The yeast *GUK1* encoding GK has been cloned in *E. coli* in laboratory scale (Konrad, 1992; Li et al., 1996). The CK and TK have been purified and characterized from the wild *S. cerevisiae* strain (Jong and Campbell, 1984; Jong et al., 1984, 1993; Liljelund and Lacroute, 1986; Ma et al., 1990). In previous studies, these kinase enzymes are extracted from bacterial sources or animal tissues and used to study the physiological and metabolic functions in the living organisms (Yan and Tsai, 1999). In this study, the yeast kinase genes from *S. cerevisiae* strain were selected as the target genes for enzyme over-expression and for production of dNDP.

In this study, pET17b plasmid vector under the control of T7 promoter was used for over-expression of the four dNMP kinases in *E. coli*. The fusion of additional amino acid residues originated from the plasmid with the target enzyme polypeptide may change special structure of the proteins and further give negative effects on their activity. In this study, the recombinant plasmids were carefully designed to produce the same enzyme as in the yeast by deleting the T7 tag encoding sequence in the upstream of all four dNMP kinase genes. The AK, GK, and TK expressed high level of soluble protein at 37°C. CK was cultured at 25°C to avoid formation of inclusion body when cultured at 37°C. In expression of all four kinases, the protein SDS–PAGE results showed that there were no significant differences in enzyme expression level when different concentrations of IPTG were used. Without using IPTG inducer, the kinase genes are fully expressed. It was found that IPTG induction was not necessary in the dNMP kinase expression, although the pET17b expression vector required the IPTG inducer according to the suppliers of the vector (Novagen).

The analyzes of the amino acid sequences of the four dNMP kinases show that a highly conserved consensus sequence, GxxxxGKxTxxxxL (GGKTL), exists near the N-terminal of the peptides for all four dNMP kinases. Since the phosphate donor, ATP, is common to all four dNMP kinases, the results may indicate that the GGKTL sequence or part of this sequence contains the NTP site of the dNMP enzymes. The conserved sequence corresponding to the second active site, the dNMP active site was not found in the amino acid sequence alignment that showed a low homology similarity level (approximately 20% similarity). The different dNMP substrate requirement for each of the four enzymes may indicate a unique amino acid sequence exists for each of the enzymes, instead of a highly conserved one in the case of the NTP site.

The $Mg^{2+}$ ion orients the phosphate donor (ATP) and the acceptor (dNMP) for phosphoryl transfer (Noda, 1973; Yan and Tsai, 1999). The previous study shows that the only the phosphate donor ATP requires the $Mg^{2+}$ in the phosphorylation reaction of NMP to nucleoside diphosphate (ADP), in which the optimal $Mg^{2+}$ concentration is equivalent to ATP concentration (Noda, 1958). However, in this study, the $Mg^{2+}$ requirement in all four dNMP kinase reactions was affected not only by ATP concentration, but also the dNMP concentration. On the other hand, the optimal pH was found as pH 8.0 for the four dNMP kinase enzyme reactions.

Up to now, no mechanism has been proposed for the dNMP kinases catalyzed phosphorylation reactions of dNMP to dNDP. In this study, a putative reaction mechanism was proposed and the kinetic parameters including the Michaelis–Menten constants and maximum reaction rate were determined. These kinetic parameters can be applied to the design and optimization of enzyme bioreactor for production of dNDP.

The proposed bioprocess for production of dNTP will provide economically competitive and environmentally friendly viable technology for the biotechnology industry and research community when compared to the chemical method currently in use. The current study paved the way for the proposed bioprocess towards the practical application. The results obtained from this study also suggest that the enzymatic method will bring about a significant economic and environmental benefits.

## NOMENCLATURE

| | |
|---|---|
| *A* | MgATP |
| *B* | deoxynucleoside monophosphates (dNMP) including deoxyadenosine monophosphate (dAMP), deoxyguanosine monophosphate (dGMP), deoxycytidylate monophosphate (dCMP), and deoxythymidine monophosphate (dTMP) |
| *E* | deoxynucleoside monophosphate kinases (dNMP kinases) including deoxyadenylate kinase (AK), deoxyguanylate kinase (GK), deoxycytidylate kinase (CK), and deoxythymidylate kinase (TK) |
| *EA* | enzyme substrate complex, E–MgATP |
| *EB* | enzyme substrate complex, E–dNMP |
| *EAB* | enzyme substrate complex, E–MgATP-dNMP |

| | |
|---|---|
| $EBQ$ | enzyme substrate complex, E–dNMP-MgADP |
| $EP$ | enzyme inhibitor complex, E–dNDP |
| $EQ$ | enzyme complex, E–MgADP |
| $EPB$ | enzyme substrate-inhibitor complex, E–dNDP-dNMP |
| $EPQ$ | enzyme substrate-inhibitor complex, E–dNMP-MgADP |
| $K_{M,A}$ | Michaelis constants of $A$ (mM) |
| $K_{M,AB}$ | Michaelis constants of the enzyme substrate complex $EA$ (mM) |
| $K_{M,B}$ | Michaelis constants of $B$ (mM) |
| $K_{M,BA}$ | Michaelis constants of the enzyme substrate complex $EB$ (mM) |
| $P$ | deoxynucleoside diphosphates (dNDP) including deoxyadenosine diphosphate (dADP), deoxyguanosine diphosphate (dGDP), deoxycytidylate diphosphate (dCDP), and deoxythymidine diphosphate (dTDP) |
| $Q$ | MgADP |
| $V_{max}$ | maximum reaction rate (μmol/min mg protein) |
| $v$ | reaction rate (mmol/min mg protein) |

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