Kinetic and Thermodynamic Characterizations of Yeast Guanylate Kinase*

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Yue Li, Yanling Zhang, and Honggao Yan‡

From the Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824

Yeast guanylate kinase was expressed at high level in Escherichia coli using pET-17b vector. It was purified to homogeneity by a simple two-column procedure with an average yield of ~100 mg/liter. The steady-state kinetic parameters for both forward and reverse reactions were determined by initial velocity measurements. The turnover numbers ($k_{cat}$) were 394 s$^{-1}$ for the forward reaction (formation of ADP and GDP) and 90 s$^{-1}$ for the reverse reaction (formation of ATP and GMP). $K_m$ values were 0.20, 0.091, 0.017, and 0.057 mM for MgATP, GMP, MgADP, and GDP, respectively. Analysis of the initial velocity patterns indicated a sequential mechanism. GMP was found to have partial substrate inhibition. The substrate inhibition was not competitive with MgATP and could be attributed to formation of the abortive complex guanylate kinase-MgADP-GMP. The equilibrium constant of the reaction was measured under various conditions by NMR and a radiometric assay. The results showed that the steady-state kinetic parameters were consistent with the thermodynamic constant. NMR titration and equilibrium dialysis showed that both substrates and products could bind to free guanylate kinase. The dissociation constants were 0.090, 0.18, 0.029, 0.084, and 0.12 mM for MgATP, ATP, GMP, MgADP, and GDP, respectively. Viscoelasticity-based kinetics was used to identify the rate-limiting steps of the reaction. The results indicated that the reaction rate is largely controlled by the chemical step.

Guanylate kinase (GK) catalyzes the reversible phosphoryl transfer from ATP to GMP in the presence of Mg$^{2+}$. The enzyme is essential for converting GMP to GDP and therefore synthesis of GTP. It plays an important role in the cGMP cycle and is also required for the metabolic activation of the antiviral drugs acyclovir and gancyclovir.

GK has been purified to various degrees of purity from several sources, but detailed characterization has been hampered by its low abundance. Mammalian GK has been characterized by steady-state kinetics, but no further mechanistic studies have been reported. It was not until 1989 that yeast GK was purified to homogeneity, and its amino acid sequence was determined by peptide sequencing. The crystal structure of yeast GK in complex with GMP was solved and refined shortly afterwards at 2 Å resolution.

The genes for yeast GK, Escherichia coli GK, and bovine GK were recently cloned. The amino acid sequence of porcine brain GK was determined by peptide sequencing. Yeast GK shares 44.8% identity with E. coli GK, 51% identity with porcine GK, and 55% identity with bovine GK. It is noteworthy that, unlike the yeast and mammalian GKs, which are monomeric, E. coli GK is tetrameric at low salt conditions and dimeric at high salt conditions. Interestingly, several proteins, including the protein encoded by Drosophila tumor suppressor gene dig-A, a rat presynaptic density protein (SAP90), a rat postsynaptic density protein (PSD-95), and the major palmitoylated membrane protein p55 of human erythrocytes, share significant homology with the entire sequence of yeast GK. It has been suggested that GK may be involved in guanine nucleotide-mediated signal transduction pathways by regulating the ratio of GTP and GDP.

In this paper we describe the heterologous expression, purification, and characterization of the wild-type yeast GK. We report the complete steady-state kinetic parameters for both forward and reverse reactions catalyzed by yeast GK. We show that GK catalyzes the phosphoryl transfer via a sequential mechanism, and the reaction rate is largely controlled by the chemical step.

**EXPERIMENTAL PROCEDURES**

Materials—All nucleotides, coupling enzymes, and phospho(enol)pyruvate were purchased from Sigma. Perdeuterated Tris was obtained from CIL. [α-32P]ATP (3,000 Ci/mmol), [8,5-3H]GDP (36 Ci/mmol), and α,β-35S-dATP (1,326 Ci/mmol) were purchased from DuPont NEN. DNA sequencing kit was obtained from U. S. Biochemical Corp. Affi-Gel blue gel was obtained from Bio-Rad.

Cloning and Expression—The gene for guanylate kinase was amplified by PCR from a yeast genomic library kindly provided by Dr. Richard Young. The primers for PCR were 5′-GGGGTACCAATATGTC-CCGTCTATCGTTTTGATATCC (forward with KpnI and NdeI sites) and 5′-CGGGATCTCATTTCCTTCTTGGAAGATATTCT-3′ (reverse with BamHI site). The PCR product was cloned into pUC19 plasmid. In order to ensure that the amplified gene is wild type, its nucleotide sequence was determined by double-stranded DNA sequencing from both forward and reverse directions. The wild-type gene was then subcloned into pET17b plasmid. The resulting expression construct was designated pET17b-YGK and transformed into the strain BL21(DE3) (16). The cloned expressing GK was selected by SDS-PAGE.

Protein Purification and Characterization—Five ml of LB medium containing 100 μg/ml ampicillin was inoculated with a single colony of the expression strain BL21(DE3) containing pET17b-YGK. It was incubated overnight at 37°C with vigorous shaking (200 rpm). The overnight culture was used to inoculate 2–4 liters of LB medium containing 100 μg/ml ampicillin. The culture was incubated at 37°C with vigorous shaking for 12–16 h. It was harvested by centrifugation. The bacterial paste was washed once with buffer A (30 mM Tris-HCl, 1 mM EDTA, pH 7.5) and kept at −70°C until use. The procedure for purification of yeast GK was modified from that of Berger et al. (6). Briefly, the frozen bacterial cells were thawed at room temperature and suspended in 100 ml of precooled buffer A. Dithiothreitol and phenylmethanesulfonyl fluoride were added to a final concentration of 1 mM. The bacterial suspension was then sonicated for 3 min in a pulse mode at 4°C. The resulting lysate was centrifuged for 30 min at 27,000 × g. The supernatant was loaded onto an Affi-Gel Blue gel column equilibrated with...
buffer A. The column was washed with buffer A until A$_{280}$ of the effluent was 0.05. It was eluted with 0.5 M GMP in buffer A. GMP fractions were identified by SDS-PAGE and concentrated to \( \sim 15 \) ml by an Amicon concentrator using a YM10 membrane. The protein solution was then applied to a Sephadex G-75 column equilibrated with buffer A. The column was developed with the same buffer. Fractions from the gel filtration column were monitored by A$_{280}$ and SDS-PAGE. Pure GK fractions were pooled and concentrated to 10–20 ml. The concentrated GK was dialyzed to remove buffer components and lyophilized. The N-terminal sequence of GK was determined by the Macromolecular Structure Facility at Michigan State University. The molecular weight was determined by MSU-NIH Mass Spectrometry Facility.

**Steady-state Kinetics**—The forward reaction kinetics was measured by a coupled assay as described by Agarwal et al. (1). Briefly, the reaction mixture contained 100 mM Tris-HCl, pH 7.7, 100 mM KCl, 5 mM MgCl$_2$, 1.5 mM phosphoenolpyruvate, 0.2 mM NADH, 40 units of pyruvate kinase, 85 units of lactate dehydrogenase, 0.3 mM ADP, and 0.6 mM GDP, 2.5 units of phosphoglycerate dehydrogenase. The reaction solution in 1 ml contained 100 mM Tris-HCl, pH 7.7, 100 mM KCl, and 5 mM dithiothreitol. GK concentration was 0.1 mM. The reaction was dialyzed to remove buffer components and lyophilized. The N-terminal sequence of GK was determined by the Macromolecular Structure Facility at Michigan State University. The molecular weight was determined by MSU-NIH Mass Spectrometry Facility.

The reaction mixture (200 µl) contained 100 mM Tris-HCl, pH 7.7, 100 mM KCl, 0.4 mM ATP, 0.4 mM GMP, 0.6 mM ADP, 0.6 mM GDP, 2.5 µCi of \( \text{[3-^2H]} \text{P}_{\text{ATP}} \), 5 mM MgCl$_2$, and 0.6 µM GK. The reaction was allowed to proceed for 5, 10, 20, and 40 min at room temperature. To terminate the reaction, 50 µl of the reaction mixture was mixed with 50 µl of 1 N HCl followed by addition of 50 µl of chloroform. After 2 min of centrifugation, 50 µl of the upper layer solution was taken and neutralized with 16 µl of 2 N NaOH. To separate the reaction products, 2 µl of the neutralized reaction mixture was loaded on a \( \times 20 \) cm TLC plate and developed with 0.8 M KH$_2$PO$_4$ solution, pH 3.4, for 1 h. After the TLC plate was dried, the nucleotides were visualized under UV light at 254 nm, and the radioactivity of ATP and ADP was analyzed by a liquid scintillation counter.

**Determination of the Dissociation Constants of Binary Complexes by Proton NMR**—Substrate titration experiments were carried out in a 5-mm NMR tube. GK was dissolved in the perdeuterated Tris buffer as described before. Proton NMR parameters were the same as for determination of the reaction equilibrium constant except those described below. The number of transients was 96 or 104. Relaxation delay was 2.8 s. The dissociation constants were obtained by nonlinear least square fit of the data to the equation

\[
\delta = \delta_0 + \frac{(\delta_1 - \delta_0)(K_a + E) + L_c - \frac{1}{2}(K_a + E + L_c)^{-1}}{2K_a E}
\]

where \( \delta_0 \) and \( \delta_1 \) are the chemical shifts of a protein resonance at the free and ligand bound states; \( \delta \) is the chemical shift of the resonance for each titration; \( E \) is the total concentration of GK; and \( L_c \) is the total concentration of the ligand. \( E \) and \( L_c \) were varied in each titration according to the following expressions:

\[
E = \frac{E_0V_0}{V_0 + \Delta V}
\]

\[
L_c = \frac{L_c0V_0}{V_0 + \Delta V}
\]

where \( E_0 \) is the initial concentration of GK; \( V_0 \) is the initial volume of the titration; \( \Delta V \) is the total volume of the added ligand solution; and \( L_c0 \) is the concentration of the ligand stock solution.

**Determination of the Dissociation Constant of GK-GDP Complex by Equilibrium Dialysis**—The experiments were carried out in a Spectrum equilibrium dialyzer. The buffer was composed of 100 mM Tris-HCl, pH 7.7, 100 mM KCl, and 5 mM dithiothreitol. GK concentration was 0.1 mM. The concentrations of GDP ranged from 0.02 to 0.64 mM. 8.5- \(^3\)H]GDP was used as a tracer for measuring bound and free GDP. Dialysis was allowed to proceed for 2, 4, and 6 h with rotation at 10 rpm at room temperature. The radioactivity of each compartment of the dialysis cells was measured by liquid scintillation counting. The data were analyzed by nonlinear least square fit to the equation

\[
L_c = \frac{P_c}{K_c + L_c}
\]

where \( P_c \) is the GK concentration, \( L_c \) and \( L_c0 \) are the concentrations of bound and free GDP, respectively.

**Viscosity-dependent Kinetics**—A concentrated sucrose stock solution was prepared in the assay buffer and then diluted to desired concentrations (10, 20, 30, and 40%). Relative viscosities of the reaction mixtures were measured by a Cannon-Fenske viscometer at 24 °C. The viscosity of the assay buffer containing 0% sucrose was used as a reference. The basic assay procedure was the same as described for steady-state kinetics. However, 50% more pyruvate kinase and lactate dehydrogenase were used. It was checked that the reaction rate was not limited by the coupling enzymes. One substrate was kept at a saturating concentration, and the other substrate was varied. GK was preincubated with the fixed substrate. The reactions were initiated with the varied substrate.

**RESULTS**

**Expression and Purification**—The gene of yeast GK has been cloned and sequenced by Konrad (9). We cloned the gene into the expression vector pET-17b by PCR from a yeast genomic library. Since Taq DNA polymerase for PCR is prone to make errors, the nucleotide sequence of the cloned gene was determined from both 5' and 3'-directions. No mutations in the gene were found in the selected clone designated pET17b-YGK. The expression construct was transformed into the \( E. \) coli strain BL21(DE3). Surprisingly, as shown in Fig. 1, GK is expressed at very high level without IPTG induction (lane 3), although it is expressed at somewhat higher level with IPTG induction (lane 4). GK was purified to homogeneity (Fig. 1, lane 5) by a simple two-column procedure modified from Berger et al. (6). The average yield is \(-100 \) mg pure GK per liter of \( E. \) coli culture. The specific activity is \(-1,500 \) U/mg. The enzyme is stable at 37 °C for at least 2 weeks. The N-terminal nine amino acid sequence was determined to be Ser-Arg-Pro-Ile-Val-Ile-Ser-Gly-Pro, in agreement with the amino acid sequence of GK.
It indicates that the initiation methionine of the recombinant GK is removed in *E. coli*. The molecular weight determined by mass spectrometry is the same as calculated without the initiation methionine (data not shown).

**Steady-state Kinetics**—The kinetics of the forward reaction (formation of ADP and GDP) was measured by coupling the reaction to those of pyruvate kinase and lactate dehydrogenase (1). The rate of the reverse reaction (formation of ATP and GMP) was measured by a coupled assay using hexokinase and glucose-6-phosphate dehydrogenase as the coupling enzymes. Kinetic parameters were obtained by nonlinear least square analysis of the initial velocity data varying both substrates. It was found that GMP has partial substrate inhibition (Fig. 2). At concentrations above 0.22 mM, the initial velocity decreases with increasing GMP concentration and levels off at ~50% of the apparent maximum velocity. The partial substrate inhibition by GMP is not competitive with MgATP. Because of the substrate inhibition the highest concentration of GMP used for full kinetic analysis was 0.17 mM. At this concentration GMP shows negligible substrate inhibition, and the kinetic data follow Michaelis-Menten equation. Double-reciprocal plots of the kinetic data are linear as shown in Fig. 3. The intersecting patterns in the double-reciprocal plots indicate a sequential mechanism for the reaction. The kinetic parameters are summarized in Table I.

**Reaction Equilibrium Constants**—In order to check whether the kinetic data are consistent with thermodynamic parameters, we determined the equilibrium constant of the reaction by NMR and a radiometric assay. We approached the reaction equilibrium from both forward and reverse directions. The proton NMR spectra of the forward and reverse reactions are shown in Fig. 4. It can be clearly seen from the spectra that there is a side reaction resulting the formation of GTP. The GTP resonances were assigned by comparing with those of authentic GTP from Sigma. Since GTP can act as a phosphate donor (albeit a poor one) with GMP as the phosphate acceptor (9), GTP is most likely generated by phosphoryl transfer between two GDP molecules. Because the side reaction is much slower than the main one, the reaction equilibrium is maintained throughout the time course once established. Thus the side reaction has no effect on the evaluation of the reaction.
Mechanism of Guanylate Kinase

Summary of steady-state kinetic parameters and viscosity effects

The uncertainties are the output of least square analysis. The standard deviations from replicate experiments were generally ~5%.

| $k_{\text{cat}}$ | $K_{(\text{cat})(\text{MgATP})}$ | $K_{(\text{cat})(\text{MgADP})}$ | $K_{(\text{cat})(\text{GMP})}$ |
|-----------------|-------------------------------|-------------------------------|-------------------------------|
| $s^{-1}$        |                               |                               |                               |
| 394 ± 15        | 0.204 ± 0.008                 | 0.080 ± 0.004                 | 0.091 ± 0.006                 |
| 90 ± 5          | 0.017 ± 0.0006                | 0.037 ± 0.002                 | 0.097 ± 0.002                 |

Viscosity effects

| $k_{\text{cat}}/K_m$ plot | $l_{k_{\text{cat}}}/k_{\text{cat}}^0$ | $K_m$ plot |
|---------------------------|---------------------------------------|------------|
| Slope                     | Intercept                             | Slope      |
| Intercept                 |                                       | Intercept  |

**A** ATP+GMP+Mg$$^2$$+GK

**B** ADP+GMP+Mg$$^2$$+GK

**C** ATP, GTP, ADP, GDP

**D** ADP, ATP, GMP, GDP

**E** ADP, ATP, GMP, GDP

**F** ADP, ATP, GMP, GDP

**G** ADP, ATP, GMP, GDP

**H** ADP, ATP, GMP, GDP

Fig. 4. Determination of reaction equilibrium constant by proton NMR. A, the initial reaction components contained 2.5 mM ATP, 2.5 mM GMP, and 5.6 mM MgCl$_2$. B, the initial reaction components contained 2.5 mM ADP, 2.5 mM GDP, and 5.6 mM MgCl$_2$. Spectra a and e were acquired before addition of GK. Spectra b and f were acquired 10 min after addition of GK. Spectra c and g were acquired 60 min after addition of GK. Spectra d and h were acquired 60 min after addition of GK.

The equilibrium constant. The reaction equilibrium was reached within 10 min after addition of GK because the equilibrium constants calculated using spectra b and f are the same as those calculated based on spectra c, d, g, and h. The equilibrium constants determined under various conditions are listed in Table II. Since the equilibrium constant, as defined under "Experimental Procedures," does not take into account the metal ion coordination states of the nucleotides, it is of no surprise that Mg$$^2$$ concentration affects the reaction equilibrium. Similar effects on the reactions catalyzed by adenylate kinase and other kinases have been observed (19, 20). The equilibrium constant determined by the radiometric assay is 2.1, in close agreement with the NMR results considering the Mg$$^2$$ effects. It is 2.2 by calculation using the kinetic parameters according to Haldane relationships. The results indicate that the kinetic parameters are consistent with the thermodynamic constant.

**Dissociation Constants of Binary Complexes**—Next we compared the kinetic parameters $K_i$ with the corresponding dissociation constants $K_{d}$. Except GK-GDP complex, the dissociation constants of all binary complexes were determined by NMR titrations. The proton NMR spectra of free GK and various binary complexes are shown in Fig. 5. Some representative NMR titration curves are shown in Fig. 6 and Fig. 7A. The curves were obtained by nonlinear least square fit of the titration data to the 1:1 binding model as described under "Experimental Procedures." Fitting the GDP NMR titration data to the 1:1 model was not satisfactory presumably because of binding of GDP to the ATP site in addition to the GMP site. The dissociation constant of GK-GDP complex was determined by equilibrium dialysis at much lower GK and GDP concentrations to minimize binding of GDP to the ATP site. The data from the equilibrium dialysis experiments could be fitted well to the 1:1 binding model as shown in Fig. 7B. The dissociation constants of the various binary complexes are listed in Table II. The $K_d$ values of all the substrate complexes except GK-MgADP are very close to those of the corresponding kinetic constants ($K_i$). The $K_d$ value of GK-MgADP is somewhat higher than that of $K_{d(MGADP)}$, presumably because of the differences in experimental conditions such as Mg$$^2$$ concentration.

**Viscosity Effects**—Viscosity-dependent kinetics was used to determine whether the chemical step is rate-limiting in the reaction catalyzed by GK. Increases in viscosity were achieved by addition of sucrose (0–40%). The results are summarized in Table I and plotted in Fig. 8. With ATP and GMP as substrates, $k_{\text{cat}}$ of the wild-type GK decreases only slightly with addition of the viscojen. Addition of sucrose has a moderate effect on $k_{\text{cat}}/K_m$. Two control experiments were performed for appropriate interpretation of the viscosity effects, one with dGMP (a poor substrate) and the other with the site-directed mutant S80A (a sluggish enzyme). With dGMP as the phosphate acceptor, $k_{\text{cat}}$ of the wild-type GK is reduced to 51 s$^{-1}$. $K_m$ for dGMP is 0.38 mM. Addition of sucrose has no effect on $k_{\text{cat}}$ and only a small effect on $k_{\text{cat}}/K_m$. With GMP as the phosphate acceptor, $k_{\text{cat}}$ of S80A mutant is 15 s$^{-1}$. $K_m$(MgGMP) and $K_m$(MgATP) of S80A are 0.50 and 0.23 mM, respectively. Addition of sucrose causes a slight decrease in $k_{\text{cat}}/K_m$ of S80A and a slight increase in $k_{\text{cat}}$.

Table I

| $k_{\text{cat}}$ | $K_{(\text{cat})(\text{MgATP})}$ | $K_{(\text{cat})(\text{MgADP})}$ | $K_{(\text{cat})(\text{GMP})}$ |
|-----------------|-------------------------------|-------------------------------|-------------------------------|
| $s^{-1}$        |                               |                               |                               |
| 394 ± 15        | 0.204 ± 0.008                 | 0.080 ± 0.004                 | 0.091 ± 0.006                 |
| 90 ± 5          | 0.017 ± 0.0006                | 0.037 ± 0.002                 | 0.097 ± 0.002                 |

The data were obtained by varying MgATP. Similar results were obtained by varying GMP.
**DISCUSSION**

GK plays an essential role in the guanine nucleotide salvage and interconversion pathway (9, 21). It has also been implicated in guanine nucleotide-mediated signal transduction pathways (12). It belongs to a family of enzymes that catalyze the phosphorylation of nucleoside monophosphates. These kinases are generally small monomeric enzymes with high substrate specificity. They are considered to be classical enzymes with substrate-induced motions. It has been suggested that the catalytic centers of these enzymes are assembled only upon substrate binding and disassembled after the products are released (22). Among the nucleoside monophosphate kinases, adenylate kinase (AK) is the most extensively studied one (23). Although the catalytic mechanism of AK is well investigated, very little is known about the catalytic mechanisms of other nucleoside monophosphate kinases. Furthermore, the structural basis of nucleotide specificity and substrate-induced fit mechanisms of these enzymes are largely unknown.

**TABLE II**

**Summary of thermodynamic parameters**

| Reaction equilibrium constants | Dissociation constants of binary complexes |
|------------------------------|------------------------------------------|
| $[\text{Mg}^{2+}] = 5.6$ mM | $K_{\text{KGMATP}}$ | $K_{\text{KATP}}$ | $K_{\text{KGMP}}$ | $K_{\text{KMGADP}}$ | $K_{\text{KGDP}}$ |
| $1.5 \pm 0.1 (n = 4)$ | $0.090 \pm 0.007^{a}$ | $0.18 \pm 0.007^{a}$ | $0.029 \pm 0.002^{a}$ | $0.084 \pm 0.009^{a}$ | $0.12 \pm 0.012^{a}$ |
| $[\text{Mg}^{2+}] = 13$ mM | $3.1 \pm 0.3 (n = 3)$ | | | | |
| $[\text{Mg}^{2+}] = 20$ mM | $5.2 \pm 0.1 (n = 2)$ | | | | |
| $[\text{Mg}^{2+}] = 45$ mM | $5.3 \pm 0.1 (n = 2)$ | | | | |

*a* Determined by NMR. The initial reaction mixture contained 2.5 mM ATP and 2.5 mM GMP. No ADP and GDP were added. The uncertainties are the standard deviations of $n$ replicate experiments. The reaction equilibrium constant determined by a radiometric assay was 2.1. The initial reaction mixture for the radiometric assay contained 0.4 mM ATP, 0.4 mM GMP, 0.6 mM ADP, 0.6 mM GDP, and 5 mM MgCl$_2$.

*b* Determined by NMR titration.

*c* The uncertainties are the output of least square analysis. The standard deviations from replicate experiments were generally $\sim 5\%$.

*d* Determined by equilibrium dialysis.

**FIG. 5.** One-dimensional proton NMR spectra of GK and its substrate complexes. A, 0.6 mM GK; B, 0.6 mM GK + 5 mM GMP; C, 0.6 mM GK + 4.2 mM ATP; D, 0.6 mM GK + 4.2 mM ATP + 6.1 mM Mg$^{2+}$; E, 0.8 mM GK + 3.6 mM ADP + 6.1 mM Mg$^{2+}$; F, 1.0 mM GK + 10.2 mM GDP.

**FIG. 6.** MgATP (A) and GMP (B) titrations measured by proton NMR. For MgATP titration, the initial solution (0.66 ml) contained 0.59 mM GK and 6 mM MgCl$_2$. The concentration of the ATP stock solution was 12.2 mM. For GMP titration (B), the initial solution contained 0.58 mM GK. The concentration of the GMP stock solution was 13.3 mM. Other experimental conditions were as described under “Experimental Procedures.”
domain of GK is very similar to that of AK; however, the GMP binding domain of GK and the AMP binding domain of AK are grossly different in structure (8). GK is rather specific with respect to nucleosidemonophosphate substrate (1, 9). The high substrate specificity of GK makes it a better model enzyme for studying the structural basis of nucleotide specificity. Moreover, GK is rather stable, in contrast to a previous report by Moriguchi (24). The high stability of GK makes it an excellent model enzyme for studying substrate-induced fit mechanisms by NMR.

Moriguchi et al. (24) have measured the $K_m$ values for MgATP and GMP (0.5 and 0.048 mm, respectively), but none of the kinetic parameters for the reverse reaction has been reported. We have determined the steady-state kinetic parameters for both forward and reverse reactions. The value for $K_m$ (GMP), determined by us, is two times that reported by Moriguchi (23), but our $K_m$ (MgATP) value is 40% of their measurement. It is noted that the specific activity of their enzyme preparation was rather low (1% of that of our preparation), and the activity was labile during storage. Thus the discrepancies could be due to the impurity in their enzyme preparation interfering with the kinetic assays and/or the possible differences in the assay procedures (no details have been given in their report). One way to check the consistency of the kinetic parameters is to measure the equilibrium constant of the reaction. The equilibrium constant calculated according to Haldane relationships is 2.2. It is in close agreement with those measured by NMR and the radiometric assay, indicating that the kinetic parameters are consistent with the thermodynamic constant.

Double-reciprocal plots of the initial velocity data show intersecting patterns for both forward and reverse reactions. The results are characteristic of a sequential mechanism. We have shown that all four nucleotides (ATP, GMP, ADP, and GDP) can bind to the free GK, and we have determined the dissociation constants of all binary enzyme substrate complexes. The results are in support of a random bi-bi mechanism. Since the dissociation constants measured by NMR and equilibrium dialysis are in line with the corresponding $K_i$ values, the kinetic mechanism is close to equilibrium random. Although an ordered mechanism cannot be ruled out yet, a random bi-bi mechanism is consistent with those of mammalian GK (1).

Partial substrate inhibition by GMP has been observed. There are several possible causes for the substrate inhibition. (i) GMP binds to the MgATP site. (ii) There is a second GMP binding site separate from the active center for activity regulation. (iii) GMP combines with the product complex GK MgADP to form the abortive complex GK MgADP-GMP. Possibility i can be ruled out because the inhibition is not competitive with MgATP. Since the binding studies indicate that GK has only one GMP binding site, possibility ii can also be ruled out. Thus possibility iii is likely to be the cause of the
and presence of viscogen, respectively (34). By combining the increase in relative viscosity, $k_5$ and $k$ are the rate constants in the absence and presence of viscogen, respectively (34). By combining the Kramer’s relationship with Equations 7 and 8, one can obtain the following normalized equations:

$$k_{cat} = k_4/k_5 + k + h) \quad (\text{Eq. 7})$$

$$k_{cat}/K_m = k_4/k_5 + k + h + k_7 \quad (\text{Eq. 8})$$

For viscosity sensitive steps, $k = k_0/\eta_{rel}$, where $\eta_{rel}$ is the relative viscosity, $k_0$ and $k$ are the rate constants in the absence and presence of viscogen, respectively (34). By combining the Kramer’s relationship with Equations 7 and 8, one can obtain the following normalized equations:

$$k_{cat}/k_0 = k_4/k_5 + k + h \quad (\text{Eq. 9})$$

$$\eta_{cat}/K_m = k_4/k_5 + k + h + k_7 \quad (\text{Eq. 10})$$

Both $k_{cat}$, $k_0$, versus $\eta_{rel}$ and $(k_{cat}/K_m)$ versus $\eta_{rel}$ plots are linear. The slopes and intercepts of the plots vary between 0 and 1 depending on the relative rates of the diffusion and chemical steps. For $k_{cat}/k_0$ versus $\eta_{rel}$ plot, the slope and intercept are determined by the relative rates of the chemical and product release steps. If the chemical step is much faster than product release ($k_5 + k_6 \gg k_7$), then the slope is 1 and the intercept is 0. When the chemical step is rate-limiting ($k_5 + k_6 < k_7$), then the slope is 0 and the intercept is 1. The ratio of the slope and intercept is equal to $(k_5 + k_6)/k_7$. On the other hand, the slope and intercept of $(k_{cat}/K_m)/k_{cat}(K_m)$ versus $\eta_{rel}$ plot are dependent on the relative rates of the chemical step and the steps both before and after the chemical step (substrate dissociation and product release). If the chemical step is much faster than substrate dissociation ($k_5 \gg k_6$) or product release ($k_6 \gg k_7$), the slope is 1 and the intercept is 0. When the chemical step is fully rate-limiting ($k_5 \ll k_4$ and $k_6 \ll k_5$), the slope is 0 and the intercept is 1. The ratio of the slope and intercept is $(k_5/k_4 + k_6/k_7)$. In the case of the wild-type GK with GMP as the phosphate acceptor, addition of the viscogen has very small effects on $k_{cat}$ (the slope and intercept of the $k_{cat}/K_m$ versus $\eta_{rel}$ plot are 0.09 and 1, respectively). It has essentially no effects on $k_{cat}$ when GMP (a poor substrate) is used as the phosphate acceptor (slope = 0.01 and intercept = 1). It causes a small increase in $k_{cat}$ in the case of the site-directed mutant S80A (a sluggish enzyme, slope = -0.1). A small increase in $k_{cat}$ with addition of a viscogen has been observed in a number of cases (27, 28, 32). It could be due to an increase in either $k_5$ or $k_6$ in the presence of the viscogen. The results suggest that product release is unlikely to be the rate-limiting step. The slope and intercept of the $k_{cat}/K_m$ versus $\eta_{rel}$ plot is 0.3 and 0.7 for the wild-type GK with GMP as the phosphate acceptor. The moderate decrease in $k_{cat}/K_m$ is significant because addition of the viscogen has only very small effects on $k_{cat}/K_m$ in both control experiments. Furthermore, addition of the viscogen has a greater effect on $k_{cat}/K_m$ than on $k_{cat}$. The results indicate that the steps before the chemical step are likely to be rate-limiting to some degree. However, the reaction rate is largely controlled by the chemical step.

In summary, a complete set of kinetic parameters has been determined for both forward and reverse reactions catalyzed by GK. The steady-state kinetic parameters are consistent with the measured thermodynamic constants. The substrate inhibition by GMP may be attributed to the formation of an abortive complex. GK catalyzes the phosphoryl transfer via a sequential mechanism. The chemical step is the major rate-limiting step in the GK-catalyzed reaction.

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