Structural and Functional Roles of Tyrosine 78 of Yeast Guanylate Kinase*

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Yanling Zhang, Yue Li, Yan Wu, and Honggao Yan‡

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

The hydroxyl group of Tyr-78 of yeast guanylate kinase (GK) is hydrogen-bonded to the phosphate of the bound GMP as revealed by x-ray crystallography. The structural and functional roles of Tyr-78 were evaluated by site-directed mutagenesis, kinetics, guanidine hydrochloride-induced denaturation, and nuclear magnetic resonance spectroscopy (NMR). Substitution of Tyr-78 with a phenylalanine resulted in a decrease in $k_{\text{cat}}$ by a factor of 131, an increase in $K_{\text{M(GMP)}}$ by a factor of 20 and an increase in $K_{\text{M(GMP)}}$ by a factor of 18. $K_{\text{M(GATP)}}$ and $K_{\text{M(GAATP)}}$ were very similar to those of the wild-type (WT) GK. The conformational stability of the mutant was lower than that of the WT by 1.0 kcal/mol as measured by guanidine hydrochloride-induced denaturation. Detailed comparison of the TOCSY and NOESY spectra of the WT GK and the mutant indicated that the conformation of Y78F is little perturbed relative to that of the WT GK at the free state and the conformation of Y78F-GMP complex is also very similar to that of the WT-GMP complex. The results taken together showed that the hydrogen bond between Tyr-78 and GMP stabilizes the GK-GMP complex by 1.7 kcal/mol, the ternary complex by 1.8 kcal/mol, and the transition state by 4.6 kcal/mol. Tyr-78 is not essential for proper folding of the enzyme but it may contribute to the conformational stability. Solvent-accessible aromatic residues were identified by using the paramagnetic probe 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl. Comparison of the free and GMP-bound forms of the WT GK by NMR indicated that there are changes in conformation and dynamics upon binding of GMP.

Guanylate kinase (GK)

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‡ To whom correspondence should be addressed. Tel.: 517-353-8786; Fax: 517-353-9334; E-mail: yan@nmr1.bch.msu.edu.

1 The abbreviations used are: GK, guanylate kinase; DQF-COSY, double quantum filtered correlated spectroscopy; GdnHCl, guanidine hydrochloride; NMP, nucleoside monophosphate; NOE, nuclear Overhauser effect; NOSEY, nuclear Overhauser enhanced spectroscopy; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; TOCSY, total correlated spectroscopy; WT, wild-type; $A_p/A$, $P^1/F^1$-diphosphadenosine 5'-pentaphosphate.

Materials—Nucleotides, coupling enzymes, and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) were purchased from Sigma. Perdeuterated Tris and l-phenylalanine (ring-D9, 98%) were purchased from CIL. Ultrapure guanidine hydrochloride (GdnHCl) and DNA sequencing kit were purchased from U. S. Biochemical Corp. Affi-Gel Blue gel was purchased from Bio-Rad.

Site-directed Mutagenesis and Protein Purification—The oligonucleotide for making the mutant Y78F was 5'-GGTAACTACTTTGGTAG-TACT-3'. The mutant was generated by the method of Kunkel (12) and selected by DNA sequencing. To ensure that there were no unintended mutations in the mutant, the entire sequence of the mutated gene was determined. Expression and purification of the mutant were the same as described for the wild-type (10) except that Y78F was eluted with 5 mM ATP in the step of Affi-Gel Blue gel chromatography.

Isotopic Labeling of the WT GK—The E. coli strain DL49PS plpS (a gift of Dr. David M. LeMaster) was used for isotopic labeling of GK. The labeling medium was prepared with l-phenylalanine (ring-D9, 98%) and other nutrients according to Muchmore et al. (13). The bacterial cells were grown in the medium containing 100 $\mu$g/ml ampicillin and 20 $\mu$g/ml chloramphenicol at 37°C. GK expression was induced by addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (final concentration) when OD$_{600}$ reached 0.8. The cells were harvested after ~3 h of further incubation. The labeled GK was purified as described previously (10).

Steady-state Kinetics—The kinetic experiments were carried out by measuring the formation of ADP and GDP using a coupled assay (1, 10). The reaction solution in 1 ml contained 100 mM Tris-HCl, pH 7.7, 100 mM KCl, 5 mM MgCl$_2$, 1.5 mM phosphonopyruvate, 0.2 mM NADH, 40 units of pyruvate kinase, 85 units of lactate dehydrogenase, 17 $\mu$g of...
Comparison of kinetic, binding, and conformational properties between WT and Y78F

**TABLE I**

|                   | Steady-state kinetics | NMR titration | Gdn-HCl denaturation$^b$ |
|-------------------|------------------------|---------------|--------------------------|
|                   | $k_{cat}/K_{M(MgATP)}$ | $K_{M(GMP)}$  | $K_{GMP}$ $^a$ | $K_{D(MgATP)}$ | $K_{D(GMP)}$ | $\Delta G_{H_2O}$ | $m$ | $D_{1/2}$ |
| WT$^a$            | 394 ± 15               | 0.20 ± 0.01   | 0.080 ± 0.004          | 0.091 ± 0.006      | 0.035 ± 0.003       | 2.0 × 10^6  | 4.3 × 10^6  |
| Y78F              | 3 ± 0.18               | 0.45 ± 0.03   | 0.16 ± 0.01           | 1.8 ± 0.2           | 0.63 ± 0.04        | 9.4 × 10^3  | 1.8 × 10^3  |

**Steady-state kinetics**

$^a$ From Li et al. (10).

$^b$ $\Delta G_{H_2O}$ is the Gibbs free energy of denaturation obtained by linear extrapolation to zero concentration of Gdn-HCl. $m$ is a measure of the dependence of the Gibbs free energy of denaturation on the concentration of Gdn-HCl. $D_{1/2}$ is the concentration of Gdn-HCl at the midpoint of denaturation.

Y78F, and varied amounts of ATP and GMP. The reaction was initiated by addition of Y78F at 25 °C. Kinetic parameters were obtained by nonlinear least square fit of the data to Cleland’s equation (14),

$$v = \frac{V_{max}[A][B]}{K_aK_b + K_a[B] + K_b[A] + [A][B]}$$  \hspace{1cm} (Eq. 1)

where $v$ is the measured initial reaction rate, $A$ and $B$ represent the two substrates (MgATP and GMP), respectively, $V_{max}$ is the maximum initial reaction rate, $K_a$ and $K_b$ are the Michaelis constants for $A$ and $B$, respectively, $K_{ab}$ is the apparent dissociation constant for the binary complex $EA$, $K_{bb}$ is the apparent dissociation constant for the binary complex $EB$ calculated using the relationship $K_{ab} = K_aK_b$.

Substrate Titration—GMP and MgATP titrations were performed on a Varian VXR 500 MHz NMR spectrometer. The methyl resonance of a methionine residue was followed by one-dimensional NMR as described previously (10). The experimental parameters are given in the legend of Fig. 2. The dissociation constants were obtained by nonlinear least square fit of the data to the equation,

$$\delta = \delta_i + \frac{(\delta_i - \delta_f)(K_a + E_i + L_i - (K_a + E_i + L_i)^2 - 4E_iL_i)}{2E_i}$$  \hspace{1cm} (Eq. 2)

where $\delta_i$ and $\delta_f$ are the chemical shifts of the protein resonance at the free and ligand bound states, $\delta$ is the chemical shift of the protein resonance for each titration, $E_i$ is the total concentration of Y78F, and $L_i$ is the total concentration of the ligand. $E_i$ and $L_i$ were varied in each titration according to the following expressions,

$$E_i = \frac{E_0V_0}{V_0 + \Delta V}$$  \hspace{1cm} (Eq. 3)

$$L_i = \frac{L_0\Delta V}{V_0 + \Delta V}$$  \hspace{1cm} (Eq. 4)

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 ligand solution added to the tube, and $L_0$ is the concentration of the ligand stock solution.

Two-dimensional Proton NMR Methods—All two-dimensional NMR experiments were performed on a Varian VXR 500 NMR spectrometer operating at a proton frequency of 500 MHz. Protein samples were prepared as described previously (10). The protein concentrations were 2–20 mM. For the paramagnetic probe experiments, TEMPO was added directly to the NMR sample tube from a 2 m TEMPOL stock solution. The ratio of TEMPOL and protein was 10 to 1. DQF-COSY (15), clean TOCSY (16–18), and NOESY (19, 20) spectra were acquired with standard pulse sequences at 27 °C. The mixing times were 45 ms for the TOCSY experiments and 200 ms for the NOESY experiments. All the two-dimensional spectra were acquired in the hypercomplex mode. The spectral width was 6000 Hz in both dimensions. The data were usually acquired with 2048 complex points in the t2 dimension and 256 complex points in the t1 dimension.
points in the t1 dimension. Spectral processing was carried out on a SGI Indigo 2 workstation using the software NMRPipe (21). The time domain data were apodized with a Gaussian function in t2 and a shifted sine bell function in t1. The t1 dimension was zero filled to 1024 points prior to Fourier transformation. The chemical shifts were referenced to internal sodium 3-(trimethylsilyl)-propionate-2,2,3,3-d4.

Equilibrium Unfolding—GdnHCl-induced unfolding was followed by fluorometry and circular dichroism (CD) measurements. Both protein and GdnHCl stock solutions were prepared in 100 mM Tris-HCl buffer, pH 7.7, containing 100 mM KCl. The concentration of the GdnHCl stock solution was determined by measuring its refractive index according to Pace (22). The final protein concentration was 0.125 mg/ml. The protein solutions were allowed to equilibrate at room temperature for ~2 h after mixing with GdnHCl. Fluorescence was measured on a Perkin-Elmer fluorometer with excitation wavelength of 282 nm and emission wavelength of 332 nm. CD spectra were obtained with a Jasco-715 spectropolarimeter using a 1-mm path length cell. Changes in ellipticity at 222 nm were followed to get a transition curve of unfolding. The unfolding data were analyzed by the linear extrapolation method (22).

The values for unfolding free energy changes were obtained by nonlinear least square analysis of the entire denaturant titration curves as described by Santoro and Bolen (23).

**RESULTS**

Kinetic and Binding Properties of Y78F—The steady-state kinetic parameters of Y78F are listed in Table I along with those of the WT GK. The effects of the point mutation on the kinetic properties of GK were rather specific. In comparison with the WT GK, the $k_{cat}$ of Y78F decreased by a factor of 131. The $K_m$(GMP) and $K_m$(MgATP) of the mutant increased by a factor of ~20. The $K_i$(MgATP) and $K_i$(MgATP) of the mutant were similar to those of the WT GK. The binding properties of Y78F were determined by one-dimensional NMR titration experiments. Representative GMP and MgATP titration curves are shown in Fig. 2. The effects of the mutation on the binding properties of GK were also specific. Thus the affinity of Y78F for MgATP was very similar to that of the WT GK, but the affinity of Y78F for GMP decreased by a factor of 12.

NMR Properties of the WT GK—The structural effects of the mutation were investigated by NMR. To use NMR to characterize the structure of a protein, one has to make sequential resonance assignment. For a protein of the size of GK (20.5 kDa), homonuclear two-dimensional NMR spectra are rather complicated. Thus our NMR analysis was focused on the better resolved aromatic spin systems. Yeast GK contains 19 aromatic residues (one histidine, one tryptophan, six tyrosines, and 11 phenylalanines). They are more or less evenly distributed among the ATP- and GMP-binding domains according to the crystal structure of the GMP complex (24). The aromatic spin systems of the WT GK were identified by a combination of DQF-COSY (data not shown) and TOCSY. The single tryptophan and all six tyrosine spin systems were easily identified by deuteration of phenylalaninyl ring hydrogens (data not shown). Of the 11 phenylalaninyl ring spin systems, 10 could be identified for the free GK and nine for the GMP complex. The missing phenylalaninyl ring spin systems could be overlapping with other aromatic spin systems or their signals are too broad to be observed in the two-dimensional correlated experiments. Surprisingly, the non-exchangeable ring protons of the histidine residue could not be identified, presumably because they were broadened somehow and overlapping with other aromatic protons. The identified aromatic spin systems are shown in Figs. 3A and 4A. The chemical shifts of the aromatic spin systems are listed in Tables II and III. The ring spin systems of Tyr-50 and Tyr-78 were sequentially assigned by comparing the TOCSY spectra of the WT GK with those of Y50F (11) and Y78F because the NMR spectra of the mutants were very similar to those of the WT GK. The other aromatic spin systems were tentatively assigned by comparing the NOE patterns of the GK-GMP complex with the crystal coordinate of the same complex (24). As shown in Fig. 5A, ~20 interresidue NOEs can be identified. There are strong intermolecular NOEs between the ring protons of Tyr-50 and Tyr-78 and H2 of the bound GMP because the distances are only ~3 Å. Yb was tentatively assigned to Tyr-77 because it has as many as six NOEs with the aromatic protons of Trp-70 (the six NOE cross-peaks could be clearly identified in the NOECSY spectrum of the GK with deuterated phenylalaninyl rings, data not shown). According to the crystal structure of the GK-GMP complex, among the aromatic residues, only the ring protons of Tyr-77 are within 5 Å distance of Trp-70. Yd was assigned to Tyr-25 because only Tyr-25 is close to two phenylalaninyl residues, namely Phe-29 or Phe-183. The NOE cross-peaks between Fb and Fd could be
attributed to Phe-29 and Phe-183 because only the two phenylalaninyl residues are close to each other and also to Tyr-25. Fe/Fj was tentatively assigned to Phe-52 or Phe-73 because it has NOE with the aromatic protons of Tyr-50 in the free GK and the aromatic protons of Tyr-78 in the GMP complex. Solvent accessibility of the aromatic residues was examined by addition of the paramagnetic probe 4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPOL). The TOCSY spectra of the free and GMP-bound WT GK in the presence of TEMPOL are shown in Figs. 3B and 4B. The degree of solvent accessibility was assessed by reductions in the TOCSY cross-peak volumes caused by TEMPOL. The results are summarized in the last columns of Tables II and III.

Comparison of the NMR Properties of the WT GK and Y78F—Substitution of Tyr-78 with a phenylalanine caused few significant changes in the chemical shifts of the aromatic protons of the free enzyme. The TOCSY spectra of the free WT GK and Y78F were almost identical (data not shown). None of the aromatic proton resonances differed by >0.05 ppm between the WT GK and the mutant. The changes in the aromatic shifts of the GMP-bound form were also limited. Only seven resonances (five residues) differed by >0.05 ppm between the WT and the mutant. The largest change in chemical shift (0.30 ppm) was found in Tyr-50. Conformational changes are best characterized by NOESY experiments. Comparison of the NOESY spectra of the WT GK and Y78F is shown in Figs. 5 (free form) and 6 (GMP-bound form). In the free form, all the interresidue NOEs except one (NOE 9) were observed in both the WT GK and Y78F. NOE 9 was missing in Y78F. The NOEs between Tyr-78 and other aromatic residues in the WT GK were replaced by the NOEs between Phe-78 and other residues in the mutant. In the GMP-bound form, all interresidue NOEs except four weak ones were observed in both the WT GK and Y78F. More importantly, the intermolecular NOEs between GK and the bound GMP were also retained in the mutant. The results indicated that the conformation of Y78F is very similar to that of the WT GK in both free and GMP-bound forms.

Conformational Stability of the WT GK and Y78F—GdnHCl-induced denaturation experiments were used to measure the conformational stability of the proteins. The unfolding reaction...
was reversible as demonstrated by refolding after complete unfolding by GdnHCl. Typical denaturation curves are shown in Fig. 7. The results were characteristic of two-state unfolding. Furthermore, the transition curves obtained by fluorometry were very similar to that obtained by CD measurements (data not shown), suggesting that the two-state model was appropriate for analysis of the data. The free energy changes of unfolding obtained by the linear extrapolation method (22, 23) are not shown, suggesting that the two-state model was appropriate for analysis of the data. The free energy changes of unfolding obtained by the linear extrapolation method (22, 23) are not shown, suggesting that the two-state model was appropriate for analysis of the data. The free energy changes of unfolding obtained by the linear extrapolation method (22, 23) are not shown, suggesting that the two-state model was appropriate for analysis of the data. The free energy changes of unfolding obtained by the linear extrapolation method (22, 23) are not shown, suggesting that the two-state model was appropriate for analysis of the data.

**TABLE II**

Chemical shifts of the aromatic residues of WT and Y78F

| Spin system | WT | Y78F | Possible residue | TEMOL accessibility |
|-------------|----|------|------------------|---------------------|
| Ya          | 6.42 | 7.24 | 6.46 | 7.23 | Tyr-77 | – |
| Yb          | 6.58 | 6.74 | 6.59 | 6.74 | B-Phe-77 | – |
| Ye          | 6.81 | 7.16 | 6.81 | 7.15 | Tyr-25 | – |
| Yd          | 6.74 | 7.45 | 6.75 | 7.46 | Tyr-50 | + |
| Ye          | 6.78 | 6.89 | 6.80 | 6.91 | Tyr-78 | + |
| Yf          | 6.64 | 7.11 | 5.41 | 6.56 | Phe-183/Phe-29 | – |
| Fb          | 6.09 | 6.88 | 6.09 | 6.90 | Phe-183/Phe-29 | – |
| Fc          | 6.24 | 6.84 | 6.20 | 6.85 | Phe-183/Phe-29 | – |
| Fd          | 6.72 | 6.99 | 6.72 | 6.99 | Phe-183/Phe-29 | – |
| Fe          | 6.83 | 6.99 | 6.84 | 7.01 | Phe-183/Phe-29 | – |
| Ff          | 7.05 | 7.34 | 7.05 | 7.32 | Phe-183/Phe-29 | – |
| Fg          | 6.91 | 7.48 | 6.93 | 7.50 | Phe-183/Phe-29 | – |
| Fh          | 6.91 | 7.48 | 6.93 | 7.50 | Phe-183/Phe-29 | – |
| Fi/Fj       | 7.10 | 7.19 | 7.12 | 7.22 | Phe-183/Phe-29 | – |
| W70         | 7.03 | 7.30 | 7.02 | 7.31 | Phe-183/Phe-29 | – |
| Ye          | 7.57 | 7.56 | 7.57 | 7.56 | Phe-183/Phe-29 | – |

* TEMPOL accessibility was determined for the WT GK. It was assessed on the basis of the peak attenuation caused by TEMPOL according to Improta et al. (36). +, ±, and – indicate that the spin system is accessible, partially accessible, or not accessible to TEMPOL.

**TABLE III**

Chemical shifts of the aromatic residues of the GMP complexes

| Spin system | WT + GMP | Y78F + GMP | Possible residue | TEMOL accessibility |
|-------------|----------|------------|------------------|---------------------|
| Ya          | 6.34 | 7.27 | 6.33 | 7.26 | Tyr-77 | – |
| Yb          | 6.62 | 6.75 | 6.57 | 6.74 | B-Phe-77 | – |
| Ye          | 6.79 | 7.16 | 6.80 | 7.16 | Tyr-25 | – |
| Yd          | 6.78 | 7.47 | 6.76 | 7.46 | Tyr-50 | ± |
| Ye          | 6.72 | 6.80 | 6.87 | 7.10 | Tyr-78 | + |
| Yf          | 6.78 | 7.31 | 6.87 | 7.10 | Tyr-78 | + |
| Fa          | 5.66 | 6.59 | 5.49 | 6.58 | Phe-183/Phe-29 | – |
| Fb          | 6.09 | 6.91 | 6.08 | 6.89 | Phe-183/Phe-29 | – |
| Fc          | 6.61 | 6.85 | 6.52 | 6.85 | Phe-183/Phe-29 | – |
| Fd          | 6.67 | 7.00 | 6.69 | 6.98 | Phe-183/Phe-29 | – |
| Fe          | 7.00 | 7.31 | 7.02 | 7.31 | Phe-183/Phe-29 | – |
| Ff          | 6.94 | 7.48 | 6.93 | 7.47 | Phe-183/Phe-29 | – |
| Fh          | 6.89 | 7.00 | 6.93 | 7.02 | Phe-183/Phe-29 | – |
| Fi/Fj       | 7.21 | 7.31 | 7.14 | 7.24 | Phe-183/Phe-29 | – |
| W70         | 7.01 | 7.29 | 7.01 | 7.29 | Phe-183/Phe-29 | – |
| Ye          | 7.58 | 7.56 | 7.57 | 7.56 | Phe-183/Phe-29 | – |

* TEMPOL accessibility was determined for the WT GK. It was assessed on the basis of the peak attenuation caused by TEMPOL according to Improta et al. (36). +, ±, and – indicate that the spin system is accessible, partially accessible, or not accessible to TEMPOL.

**DISCUSSION**

GK belongs to the family of NMP kinases. Among NMP kinases, adenylate kinase has been most extensively studied (25). High resolution crystal structures have been reported for adenylate kinase, GK, and UMP/CMP kinase (24, 26–32). The ATP-binding sites of all known structures are highly conserved. The AMP-binding site of adenylate kinase and the UMP/CMP-binding site of UMP/CMP kinase are also very similar. On the other hand, the chain fold of the GMP-binding domain of GK is grossly different from those of the NMP-binding domains of adenylate kinase and UMP/CMP kinase (24). The AMP-binding domain and the UMP/CMP-binding domain are completely α-helical. In contrast, the GMP-binding domain is composed of a four-stranded β-sheet and only a short helix (Fig. 1). While only two arginine residues are involved in binding the phosphate moiety of the NMP in adenylate kinase or UMP/CMP kinase, the phosphate moiety of the bound GMP in GK interacts with a pair of tyrosine residues as well as a pair of arginine residues. In this study, we have focused on the structural and functional roles of Tyr-78. Substitution of Tyr-78 with a phenylalanine caused a decrease in $k_{cat}$ by a factor of 131 and increases in $K_m$ and $K_i$ for BMP by a factor of $\approx 20$, and little changes in $K_m$ and $K_i$ for MgATP. The mutation also resulted in a decrease in the stability of the enzyme by $\approx 1.0$ kcal/mol.

Since the changes in the kinetic properties and conformational stability could be due to global structural perturbations, we have compared the conformation of the mutant with that of the WT GK by NMR. Detailed analysis of the two-dimensional spectra of the aromatic protons has indicated that there are only a few small changes (<0.05 ppm) in the chemical shifts in the mutant spectra at the free state, and all the interresidue
NOEs except a weak one are retained. The results suggest that Y78F is appropriately folded and its conformation is highly similar to that of the WT GK. The decrease in conformational stability is not likely the result of global structural changes.

Comparison of the two-dimensional spectra of the GMP-bound forms has indicated that there are some large changes in the chemical shifts (Tyr-50 and Fa, 0.1 ppm). Since both Tyr-78 and Tyr-50 are hydrogen-bonded to the phosphate of GMP (24), the chemical shift changes of Tyr-50 are likely due to changes in the local electronic environment. Substitution of Tyr-50 with a phenylalanine also causes large changes in the chemical shifts of the aromatic protons of Tyr-78 at the GMP-bound state (11). However, replacement of Tyr-50 or Tyr-78 with a phenylalanine does not alter the chemical shifts of the other residue in the free form, indicating the two tyrosine residues do not interact at the unliganded state. The causes for the chemical shift changes of other residues are not obvious. Some minor changes in the chemical shifts are likely due to small differences in the experimental conditions such as pH and degree of GMP saturation. Most interresidue NOEs observed in the NOESY spectrum of the WT complex are also observed in the NOESY spectrum of the mutant complex. Furthermore, the substi-

**Fig. 5.** NOSEY spectra of the aromatic protons of WT GK (A) and Y78F (B). Interresidue NOEs are numerically labeled. In the WT GK, 1–3 and 16 are the NOEs between Fb and Fd; 4 is the NOE between Fb and Yd; 5 and 6 are the NOEs between Fc and Fe; 7 is the NOE between Fa and Fe; 8, 10–12, 17, and 18 are the NOEs between Yb and Trp-70; 9 is the NOE between Yb and Tyr-78 or Fi; 13, 14, and 19 are the NOEs between Tyr-50 and Tyr-78; 15 is the NOE between Fi and Tyr-78; 20 is the NOE between Fb and Fi; 21 is the NOE between Fi and Yd. In Y78F, 13, 14, and 19 are the NOEs between Tyr-50 and Phe-78; 15 is the NOE between Fi and Phe-78; all others are the same as in the WT GK.

**Fig. 6.** NOSEY spectra of aromatic protons of WT GK (A) and Y78F (B) both in complex with GMP. Interresidue NOEs and the NOEs between the bound GMP and the aromatic residues are numerically labeled. In the WT complex, 1, 3, 4, 10, and 11 are the NOEs between Fb and Fd; 2, 5, and 15 are the NOEs between Fb and Yd; 6 is the NOE between Fb and Ya; 7–9, 14, and 16 are the NOEs between Yb and Trp-70; 12 and 17 are the NOEs between Fi and Tyr-50; 13 is the NOE between Tyr-50 and Tyr-78; 18 is the NOE between Fc and Fj; 19 is the NOE between Fd and Yd; 20 is the NOE between Fi and His-8 of GMP; 21 is the NOE between Tyr-50 and His-8 of GMP; all others are the same as in the WT complex.
We have reported the characterization of the structural and functional roles of Tyr-50 (11). The hydroxyl group of Tyr-50 is also hydrogen-bonded to the phosphate of GMP with similar geometry. In comparison with Try-50, Tyr-78 contributes less to the stabilization of the binary and ternary complexes by -0.5 kcal/mol. This is consistent with the crystallographic observation that the hydrogen bond between Tyr-78 and the phosphate of GMP is slightly longer and therefore a bit weaker than that between Tyr-50 and the phosphate of GMP. However, compared with Try-50, Tyr-78 contributes more to the transition state stabilization by 1.4 kcal/mol and therefore is more important for catalysis. This suggests that the hydrogen bond between Tyr-78 and GMP becomes stronger than that between Tyr-50 and GMP as the reaction progresses to the transition state.

Substrate-induced fit is believed to be the mechanism by which kinases use to avoid hydrolytic activity (33). It has been suggested that the active centers of NMP kinases are assembled with large domain movements upon binding of both substrates (31). GK is also expected to undergo substrate-induced movements (24). Because of its small size and stability, GK is an excellent model system for detailed analysis of the mechanisms of substrate-induced fit by NMR. Binding of GMP causes significant changes (>0.05 ppm) in the chemical shifts of a number of aromatic spin systems (Tyr-50, Trp-70, Tyr-78, Ya, Fb; Tyr-50 and Fi/Fj; Fc and Fi/Fj) (Figs. 2A and 3A). Fe changes so dramatically that it cannot be identified in the spectra of the complexed form. The changes in the chemical shifts of Tyr-50 and Tyr-78 are likely caused by hydrogen bonding to the phosphate of the bound GMP and the ring current effects of GMP. The chemical shift changes of other residues may reflect conformational changes induced by binding of GMP, although small chemical shift changes may be due to slight variations in the experimental conditions. These chemical shift changes are unlikely due to ring current effects because all aromatic residues except Tyr-50 and Tyr-78 are ≈9 Å away from the guanine moiety of GMP. The interpretation is supported by the NOESY data and the paramagnetic probe TEMPO. Comparison of the NOESY spectra of the free and GMP-bound forms (Figs. 5A and 6A) shows that most NOEs are identical between the two forms of GK. However, there are three pairs of residues (Yb and Fi/Fj; Tyr-78 and Fi/Fj; Fc and Fd) with NOEs in the free form but not in the GMP-bound form and another three pairs of residues (Ya and Fb; Tyr-50 and Fi/Fj; Fe and Fi/Fj) that show NOEs in the GMP-bound form but not in the free form. In contrast, comparison of the NOESY spectra of the WT GK and Y78F reveals that only a few NOEs (one in the free form and four in the GMP-bound form) become very weak or disappear in the mutant spectra. No additional NOE peaks appear in the mutant spectra. The results suggest that there are conformational changes upon binding of GMP. However, the conformations of the free form and the GMP-bound form are very similar. This is characteristic of the domain movements resulting from hinge and shear motions (34).

Paramagnetic probes have been used to aid identification of surface residues by NMR (35–38). Addition of the paramagnetic probe TEMPOL resulted in significant attenuation of many cross-peaks (Figs. 3B and 4B). However, it did not cause any significant changes in their chemical shifts, indicating that TEMPOL does not bind to GK. Furthermore, qualitatively, TEMPOL accessibility determined for the GMP complex is consistent with the solvent accessibility calculated from the crystal structure coordinate (24). Thus the attenuation of the cross-peaks is likely due to the exposure of the side chains to TEMPOL but not specific binding. Binding of GMP brings protection of several aromatic residues from the paramagnetic

![Fig. 7. GdnHCl-induced unfolding of the WT GK (A) and Y78F (B) measured by fluorometry. The dotted lines were obtained by nonlinear least-square fit of the data as described under “Experimental Procedures.”](image-url)
bleaching, including Tyr-50 and Tyr-78. However, Fe becomes more accessible to TEMPO-b in the presence of GMP. As mentioned earlier, only Tyr-50 and Tyr-78 are in close vicinity of the bound GMP. Thus the changes in the TEMPO accessibility upon binding of GMP are likely due to substrate-induced changes in conformation and/or dynamics. Müller et al. (30) have recently determined the crystal structure of free E. coli adenylyl kinase. Comparing it with the crystal structure of the same enzyme in complex with Ap5A, a bisubstrate analogue, they have noticed significant differences in the distribution of B-factors between the two structures. On the basis of B-factors, they have suggested that the lid and AMP-binding domains are mobile in the free form and become immobilized upon binding of Ap5A. On the other hand, two loops (α4-β3 and α5-β4) are fixed in the free form and become mobile upon binding of Ap5A. They have proposed that the two mobile loops are the “energetic counterweight” that prevent the enzyme from being trapped in an energetic well. It is of great interest to see how substrate-induced conformational and dynamic changes in GK correlate with catalysis.

In conclusion, we have shown that the hydrogen bond between hydroxyl group of Tyr-78 and the phosphate of GMP is critical for binding of GMP and catalysis. It stabilizes the GK-GMP complex by 1.7 kcal/mol, the ternary complex by 1.8 kcal/mol, and the transition state by 4.6 kcal/mol. Tyr-78 is not essential for binding of GMP and catalysis. It stabilizes the intermediate hydroxyl group of Tyr-78 and the phosphate of GMP is changes in GK correlate with catalysis.

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