Mechanism of Adenylate Kinase. What Can Be Learned from a Mutant Enzyme with Minor Perturbation in Kinetic Parameters?†

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ABSTRACT: The structural and functional roles of threonine-23 in the chicken muscle adenylate kinase (AK) were investigated by site-directed mutagenesis coupled with proton nuclear magnetic resonance (NMR) and phosphorus stereochemistry. The residue is potentially important because it is conserved among all types of AK and is part of the consensus P-loop sequence, ^{15}GXPGXGKGT^{23}. A mutant enzyme T23A (replacing threonine-23 with alanine) was constructed. Analyses of conformational stability and proton NMR indicate that the side chain of this residue contributes little to the structure of AK, which suggests that the side chain of Thr-23 does not play a structural role. The steady-state kinetic data of the mutant enzyme T23A showed no change in k_{cat} and only 5–7-fold increases in K_m and dissociation constants. Such minor changes in kinetic data are insufficient to suggest a functional role of Thr-23. However, two-dimensional NMR analyses of WT·MgAP$_5$A and T23A·MgAP$_5$A complexes indicated that the side chain of Thr-23 is in proximity to the adenine ring of the ATP moiety in the WT·MgAP$_5$A complex in solution. In addition, T23A showed a significant perturbation in the stereospecificty toward the diastereomers of (R_p)- and (S_p)-adenosine 5′-(1-thiotriphosphate) (ATPαS), with the R_p/S_p ratio increased from <0.02 in wild-type to 0.37 in T23A. Detailed ^{31}P NMR analysis indicated that the stereospecificity at the AMP site was not perturbed. These results suggest that the side chain of Thr-23 is involved in catalysis, most likely via a hydrogen bonding interaction Thr–OH···O–P$_\alpha$(ATP). Thus, even though it is not obvious from the kinetic data of the mutant T23A, the side chain of Thr-23 does interact directly with ATP during catalysis. The significance of these results is discussed in relation to the structure–function relationship of adenylate kinase in particular and the use of site-directed mutagenesis in the study of enzyme mechanism in general.

Although site-directed mutagenesis has made an enormous contribution in advancing the knowledge of the structure–function relationship of proteins and enzymes, the conservatives in the field have always been concerned with proper interpretation of the functional data of site-specific mutants. The earliest voice came from Gerlt, who rightfully pointed out that the perturbation in the kinetic property of a mutant enzyme should not be interpreted in functional terms unless it can be demonstrated that the global conformation has not been perturbed (Hibler et al., 1987). As an extention of this cautionary note, we have advocated that one should examine the conformation of not only the free enzyme, but also enzyme-substrate complexes (Yan et al., 1990a; Tsai & Yan, 1991). However, no matter how rigorous one may be in examining the structures, there is always a difficulty in relating structural perturbations to functional changes: a major structural change may not necessarily be responsible for the perturbation in function, whereas a minor conformational perturbation near the active site could have substantial impact on the activity.

There are different concerns when the kinetic constants are not perturbed or only slightly perturbed. The straightforward interpretation would be that the mutated residue is not involved in catalysis. However, when this happens to a highly conserved residue, or to a residue whose proximity to substrate has already been demonstrated by X-ray crystallography, one wonders what to believe. It can be argued that highly conserved residues do not necessarily play important structural or functional roles and that a residue in proximity to a substrate does not necessarily contribute to catalysis (Tsai & Yan, 1991). In some cases it has been demonstrated that the functional role of the mutated residue is replaced by a nearby residue, but this is not always the case (Sekharudu et al., 1992).

In this paper we address the question of "what can be learned if mutation of a potentially important residue leads to only minor changes in kinetic constants" using the T23A mutant of adenylate kinase (AK,[1] from chicken muscle,[2] overexpressed in *Escherichia coli*) as an example. Thr-23 is a potentially important residue for two reasons: (i) It corresponds to the conserved Thr/Ser in the broadly defined P-loop sequences for various enzyme families: GXXXXGK[TS] (Saraste et al., 1990). In the case of AK the consensus P-loop sequence

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; ADPαS, adenosine 5′-(1-thiodiphosphate); AK, adenylate kinase; AMP, adenosine 5′-monophosphate; AMPS, adenosine 5′-monothiophosphate; AP$_5$A, P^1,P^5-bis(5′-adenosyl)pentaphosphate; ATP, adenosine 5′-triphosphate; ATPαS, adenosine 5′-(1-thiotriphosphate); CD, circular dichroism; COSY, correlation spectroscopy; 1D, one-dimensional; 2D, two-dimensional; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; FID, free induction decay; GB, Gaussian broadening; Gdn-HCl, guanidine hydrochloride; LB, line broadening; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhanced spectroscopy; PAGE, polyacrylamide gel electrophoresis. SDS, sodium dodecyl sulfate; TOCSY, total correlated spectroscopy; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; WT, wild-type.

[2] The AK from different sources are abbreviated as follows: from muscle, AK1 (followed by letters c, h, p, and r designating chicken, human, porcine, and rabbit, respectively); from *E. coli*, AKe; from yeast, AKy. Unless otherwise specified, the numbering system used in this paper is the conventional system for AK1. Although AK1c has one additional residue near the N-terminal (Kishi et al., 1986), the Met-1 residue is absent in the AK1c expressed in *E. coli* (Tanizawa et al., 1987). This makes numbering of AK1c consistent with other AK1.

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is ^{15}GXPGXGKGT[23]. The only two residues possessing a functional side chain in this ubiquitous "glycine-rich" loop are the lysine and the serine/threonine. The functional role of the conserved lysine in this motif has received very extensive attention (Tian et al., 1990; Reinstein et al., 1990) whereas that of the latter has been largely ignored. (ii) The side chain of Thr-23 has been shown to be in contact with the adenosine of the ATP moiety in the crystal structure of AKy·MgAP$_5$A (Egner et al., 1987).

We first showed that the T23A mutant is only minorly perturbed in kinetic constants and is not perturbed in structure. Wondering whether Thr-23 is indeed located in proximity to ATP in our system, we undertook detailed proton NMR analysis to demonstrate that this is indeed the case. A tempting conclusion at this point would be that Thr-23 is not functional despite its proximity to ATP. However, we further pursued this issue with phosphorus stereochemistry and found that the stereospecificity at the α-position of ATP was substantially perturbed in T23A. The results allowed us to conclude that the hydroxyl group of Thr-23 interacts with the α-phosphate of ATP during catalysis, which agrees with the refined crystal structure published during the course of this work (Müller & Schulz, 1992). In addition, since all of the previous evidence for the location of the MgATP site is based on physical studies of the AK·MgAP$_5$A complexes of large variants of AK, our results support that the ATP moiety in the AK·MgAP$_5$A complex of a small variant of AK (AK1) represents the true MgATP site under catalytic conditions. An important lesson to be learned from this work is that mutation of a functional residue may not necessarily lead to a large perturbation in kinetic constants.

MATERIALS AND METHODS

Materials. The oligonucleotide used for the construction of mutant T23A, GGGAAGGGGGCGCAATGCGA, was synthesized at the Biochemical Instrumental Center of the Ohio State University. DNA sequencing and mutagenesis kits were purchased from United States Biochemicals and Amersham, respectively. Perdeuterated Tris was purchased from MSD Isotopes. Guanidine hydrochloride was purchased from Schwarz Mann Biotech. ADP, AMP, AMPS, AP$_5$A, ATP, coupling enzymes, and other reagents were obtained from Sigma. Other phosphorothioate analogues were synthesized as described by Dahnke et al. (1992).

Construction and Purification of T23A. The mutant T23A was constructed by using the mutagenesis kit from Amersham. The procedure described in the manual provided by the manufacturer was followed without modification. The full-length AK gene was sequenced by the dideoxy nucleotide terminating method to ensure that no undesirable mutations had occurred. The method used to purify mutant T23A was essentially the same as that described by Tian et al. (1988) for WT AK, except that the linear elution gradient for the phosphocellulose P-11 column was 50–300 mM. The purity of the enzyme was examined by SDS-PAGE with silver staining on a PhastSystem.

Steady-State Kinetics. The kinetic experiments were carried out at 30 °C and pH 8.0 by monitoring ADP formation with pyruvate kinase/lactate dehydrogenase as the coupling system (Rhoads & Lowenstein, 1968). The values of k_{cat}, K (Michaelis constant), and K_i (dissociation constant) were obtained by varying both MgATP and AMP concentrations followed by analyses according to the equation of Cleland (1986) for a random Bi Bi system. The details have been described previously (Tian et al., 1988).

Proton NMR Methods. All proton NMR experiments were performed on Bruker AM 500 spectrometer at 27 °C unless otherwise specified. Chemical shifts were referenced to internal sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4. Sample preparation and 1D NMR titration experiments were essentially the same as described by Yan et al. (1990a). All the samples were adjusted to pH 7.5 except those for 1D NMR titration experiments, which were adjusted to pH 7.8. The enzyme concentrations for 1D and 2D experiments were about 1 and 2 mM, respectively. In the 1D NMR titration experiments, 16K points were collected and zero-filled to 32K. Generally, 300–500 transients were collected at each ligand concentration. The dissociation constants K_d were obtained by nonlinear least-squares fitting of the chemical shift values as a function of ligand concentration. Three resonances were monitored and the K_d values thus obtained were averaged to give the final K_d.

Standard pulse sequences and phase cycling were used for all 2D NMR experiments: NOESY (Bodenhausen et al., 1984), COSY (Marion & Wüthrich, 1983), and TOCSY (Bax & Davis, 1985). All spectra were obtained in the phase-sensitive mode (Marion & Wüthrich, 1983). Spectral widths in all experiments were 5300 Hz in both dimensions. Generally, a 4K × (400–512) time domain matrix was recorded. In COSY experiments, the two-dimensional data matrix was multiplied with an unshifted sine bell weighting function in both dimensions. In NOESY and TOCSY experiments, the two-dimensional data matrix was multiplied by a shifted sine bell function (SSB1 = 12) in t_1 and by a Gaussian function (LB = –3, GB = 0.1) in t_2. Then the data matrix was zero-filled to 4K × 2K matrix prior to Fourier transformation. The mixing time in NOESY experiments was 200 or 120 ms and in TOCSY experiments 40 ms.

Phosphorus NMR Methods. All phosphorus NMR experiments were performed on a Bruker AM 300 NMR spectrometer at 30 °C. Chemical shifts were referenced to external 85% H_3PO_4. A 45° pulse and a 0–1.0-s relaxation delay were used in data acquisition. All spectra were broadband proton-decoupled with the WALTZ sequence (Shaka et al., 1983). The spectral width was 75 ppm, and 32K data points were recorded for each spectrum with the quadrature data mode. 1000–2000 transients were obtained for each spectrum. All spectra were processed with Gaussian multiplication (LB = –2, GB = 0.2).

Gdn-HCl Induced Denaturation. The denaturation of the enzyme induced by Gdn-HCl was measured by following the changes at 222 nm of the circular dichroism (CD) spectra obtained on a JASCO-500C polarimeter. The concentration of the stock solution of the Gdn-HCl in distilled H_2O was calculated from its refractive index (Nozaki, 1972). A concentrated stock solution of Tris and KCl (pH 8.0) was added to the above solution to reach the final concentration of 75 mM Tris and 65 mM KCl. The pH was then adjusted and the final concentration of the Gdn-HCl was corrected by the dilution effect. A concentrated enzyme stock solution (1–2 mg/mL) was prepared by dissolving the enzyme in 75 mM Tris buffer (pH 8.0) containing 65 mM KCl and 1 mM DTT. Insoluble material was removed by centrifugation. The enzyme, buffer, and Gdn-HCl were mixed in a quartz cuvette to a total volume of 1.2 mL and allowed to equilibrate at 25 °C for 10 min. Spectra were baseline corrected. The $\Delta G_d^{H_2O}$ and m values were calculated as described in the footnote of Table I (Pace, 1986).

RESULTS

Kinetic Constants of T23A Are Only Slightly Perturbed. As shown in Table I, the k_{cat} of T23A is similar to that of WT, the dissociation constants (K_i) increase by only 4–5-fold, and

Table I: Comparison of Kinetic and Conformational Properties between WT and T23A

parameters	unit	WT[a]	T23A
	Steady-State Kinetics[b]		
k_{cat}	(s^{-1})	650	755
$K_{(MgATP)}$	(mM)	0.042	0.31
$K_{(AMP)}$	(mM)	0.098	0.60
$K_{i(MgATP)}$	(mM)	0.16	0.76
$K_{i(AMP)}$	(mM)	0.37	1.47
$k_{cat}/K_{(MgATP)}$	(M^{-1} s^{-1})	1.55×10^7	0.24×10^7
$k_{cat}/K_{(AMP)}$	(M^{-1} s^{-1})	0.66×10^7	0.13×10^7
	Conformational Stability[c]		
$\Delta G_d^{H_2O}$	(kcal/mol)	4.8	5.1
$D_{1/2}$	(M)	0.79	0.81
m	(kcal/mol·M)	6.1	6.3

[a] The kinetic data for WT are from Tian et al. (1990). [b] Obtained by varying the concentration of both substrates and analyzed according to Cleland (1986). The error limits are estimated to be 5% for k_{cat} and ±10% for K and K_i values. [c] The data were analyzed according to the equation $\Delta G_d = \Delta G_d^{H_2O} - m$[Gdn-HCl] (Pace, 1986), where ΔG_d is the Gibbs free energy of denaturation at various concentrations of Gdn-HCl, $\Delta G_d^{H_2O}$ is that extrapolated to zero concentration of Gdn-HCl, and m is a constant. $D_{1/2}$ is the concentration of Gdn-HCl at the midpoint of unfolding. The error limit for $\Delta G_d^{H_2O}$ is estimated to be ±0.3 kcal/mol.

the Michaelis constants (K) increase by only 6–7-fold. The increases in K_i and K values are too small to be considered significant; in addition, they are *not* specific to MgATP and thus may not reflect specific interactions between Thr-23 and MgATP. The lack of change in k_{cat} suggests that the chemical step has not been perturbed since it has been shown that the chemical step is nearly rate-limiting in the catalysis by WT AK (Tian et al., 1990). Thus, although the T23A mutant does not behave exactly like WT AK, the kinetic data are only minorly perturbed and are insufficient to suggest a role of Thr-23 in interacting with MgATP as suggested by the crystal structure in Egner et al. (1987).

The above kinetic analysis was based on the random Bi Bi mechanism established for WT AK (Rhoads & Lowenstein, 1968) and the fact that the chemical step is nearly rate-limiting. If the mutant enzyme deviates from such properties, then the K_i and K values may deviate from the real dissociation constant (K_d) and Michaelis constant (K_m), respectively. To rule out the possibility that the kinetic mechanism has been perturbed in the mutant, K_m values were also determined by saturating either substrate. The K_m values thus obtained for T23A are 0.28 mM for MgATP when AMP was held at 2.5 mM and 0.43 mM for AMP when MgATP was held at 4.0 mM. These values agree well with those listed in Table I obtained by varying both substrates. The dissociation constant of AMP was also determined by NMR titration experiments. The K_d thus obtained was 0.4 mM, which is lower than the K_i value but is close to the value of WT (0.5 mM, from Sanders et al., 1989). The reason for the 4-fold difference between the K_d and K_i of AMP is unclear, but it is likely to be caused by the different nature of the two experiments.

The Conformation of T23A Is Not Perturbed. As shown in Figure 1, the 1D proton NMR spectra of T23A and its complexes with AMP, ATP, and $MgAP_5A$ are similar to those of WT, but there are some minor differences. For a more quantitative assessment of conformational perturbations, we performed NOESY and TOCSY experiments and identified the aromatic spin systems for the T23A complex of $MgAP_5A$. As shown in Table II, only three histidine resonances (those underlined) differ by >0.02 ppm between WT and T23A. Since these histidine resonances have been shown to be very sensitive to pH (Yan & Tsai, 1991), the observed deviations are likely to be caused by a small difference in pH between the two samples. This result indicates that there is little

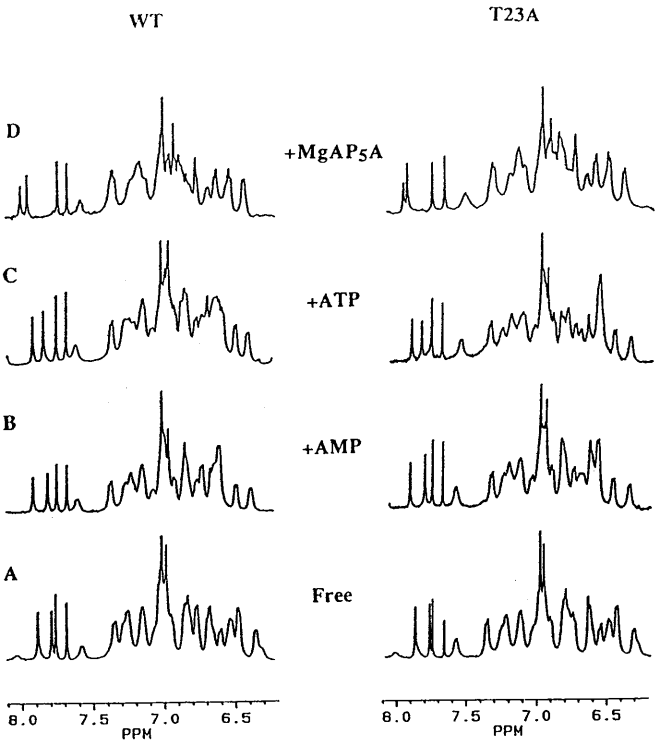

FIGURE 1: Proton NMR spectra of the aromatic protons of WT (left) and T23A (right). The spectra of WT are reproduced from Sanders et al. (1989). The conditions for the right panel are as follows: (A) 1.46 mM T23A; (B) 1.46 mM T23A + 18.8 mM AMP; (C) 0.77 mM T23A + 2.54 mM ATP; (D) 1.8 mM T23A + 2.0 mM $MgAP_5A$. The spectra were obtained at pH 7.8, 27 °C, and processed with Gaussian multiplication (LB = –5, GB = 0.1).

Table II: Chemical Shifts (ppm) of the Aromatic Spin Systems of the $MgAP_5A$ Complexes (pH 7.5)[a]

spin system	WT + $MgAP_5A$			T23A + $MgAP_5A$		
Fa	6.40	6.54	6.68	6.40	6.55	6.68
Fb	6.43	7.00	7.16	6.42	7.00	7.16
Fc	6.80	6.95	6.99	6.80	6.95	7.00
Fd	6.93	7.18	7.22	6.93	7.18	7.23
Fe	7.00	7.16	7.57	7.01	7.16	7.55
Yb	6.51	6.62		6.52	6.62	
Yd	6.75	7.02		6.77	7.04	
Ye	6.85	7.12		6.85	7.12	
Yf	6.90	7.36		6.90	7.36	
Yh	6.95	7.34		6.95	7.34	
Ha	6.98	7.64		6.98	7.68 (+0.04)	
Hb	6.89	7.69		6.92 (+0.03)	7.77 (+0.08)	
Hc	6.83	7.99		6.83	7.98	
Hd	6.76	7.94		6.75	7.95	

[a] The underlined values are the resonances that differ from WT by more than 0.02 ppm, and the magnitudes of the differences are shown in parentheses.

conformational perturbation in the mutant enzyme at different stages of catalysis. The conformational stability of T23A is also unperturbed relative to WT, as shown by the free energy of denaturation, $\Delta G_d^{H_2O}$, also listed in Table I. These results suggest that Thr-23 does not play an important structural role at the different stages of catalysis.

The Rationale behind Further NMR Analysis. One of the possible explanations for the lack of significant perturbation in the kinetic constants of T23A is that even though it is in proximity to ATP in the crystal structure of AKy·$MgAP_5A$ (Egner et al., 1987), the binding mode of $MgAP_5A$ is different in our system due to two possible factors: a difference between crystal and solution structures, and/or a difference between large and small variants of AK. The latter is of particular concern since the ATP moiety in AKy is surrounded by a

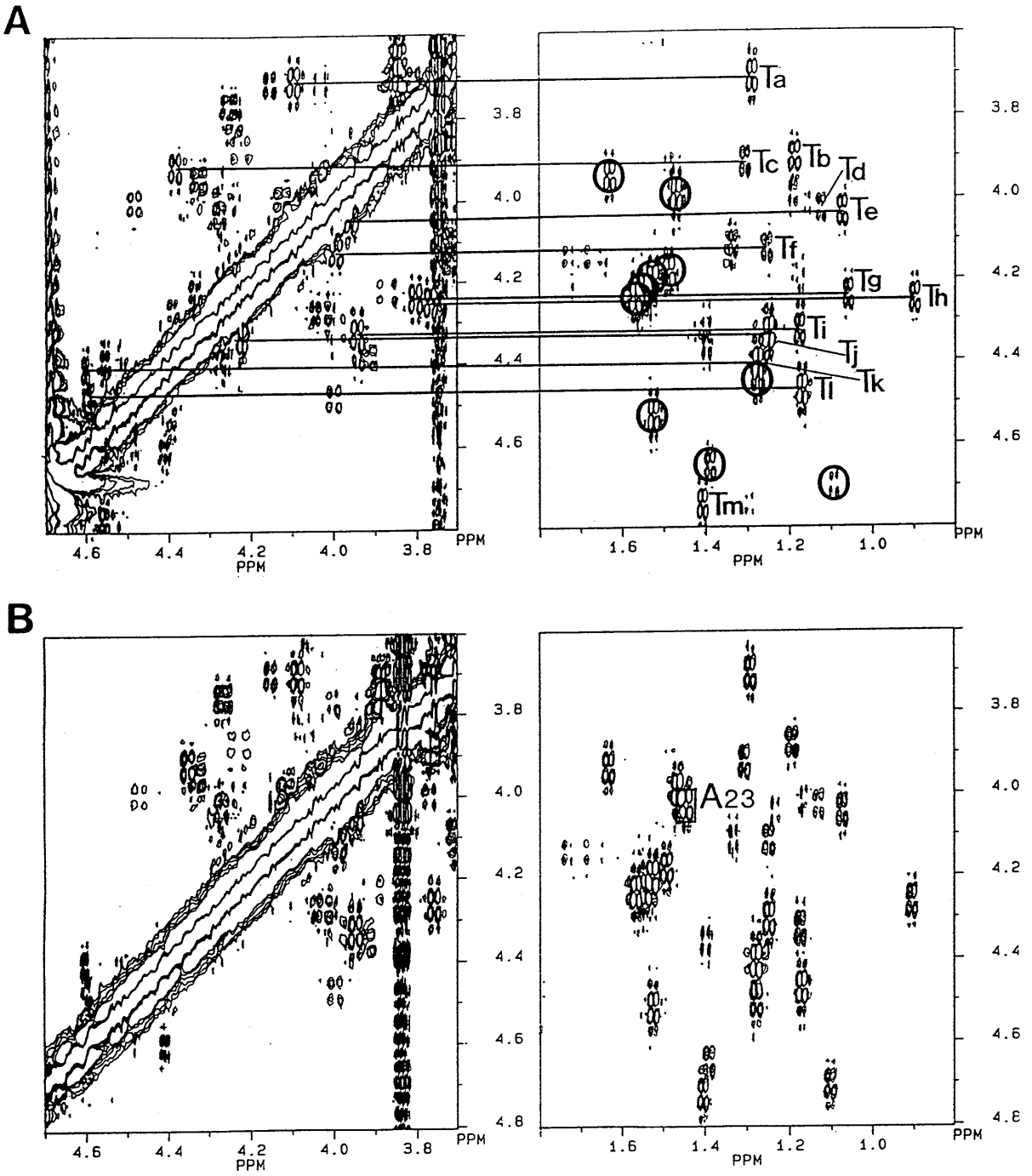

FIGURE 2: COSY spectra of WT·$MgAP_5A$ at pH 7.5, 34 °C (A) and T23A·$MgAP_5A$ at pH 7.5, 27 °C (B) in ^2H_2O. In (A) the spin systems of the 13 threonine residues are labeled as Ta-Tm, while those of the 10 alanine residues are circled. In (B) only the newly arised cross peak due to Ala-23 is marked by a square.

30-residue insertion segment which is absent in AK1. We therefore carried out solution structural studies of AK1 to establish the proximity between Thr-23 and the ATP moiety of $MgAP_5A$ as described in the following sections.

Assignments of the Resonances of Thr-23 and Ala-23 in AK·$MgAP_5A$. We were able to identify the side chain spin systems of all 13 Thr residues in WT·$MgAP_5A$ on the basis of the COSY, TOCSY, and NOESY spectra. The identified Thr residues are labeled as Ta–Tm in the COSY spectrum of WT·$MgAP_5A$ (Figure 2A). Also identified in this figure are the spin systems of all of the ten alanine residues, as indicated by circles.

Comparison of the COSY spectrum of T23A·$MgAP_5A$ (Figure 2B) with that of WT·$MgAP_5A$ in Figure 2A indicates that all but 1 (Tg) of the 13 threonine spin systems identified in Figure 2A can also be found in Figure 2B. In addition, a new cross-peak labeled as A23 appears in Figure 2B, which can be identified as the α-proton/β-proton cross peak of the new alanine residue. These results unequivocally establish that the spin system of Tg corresponds to that of Thr-23. The chemical shifts of the side chain protons of Thr-23 in WT·$MgAP_5A$ and those of Ala-23 in T23A·$MgAP_5A$ are listed in Table III.

Table III: Chemical Shifts (ppm) of Thr-23 and Ala-23 in AK·$MgAP_5A$ Complexes

residue	H_α	H_β	H_γ
Thr-23 in WT·$MgAP_5A$	3.80	4.24	1.05
Ala-23 in T23A·$MgAP_5A$	4.04	1.44	

Assignments of Nucleotide Resonances in AK·$MgAP_5A$. To examine whether there is a NOE between Thr-23 and

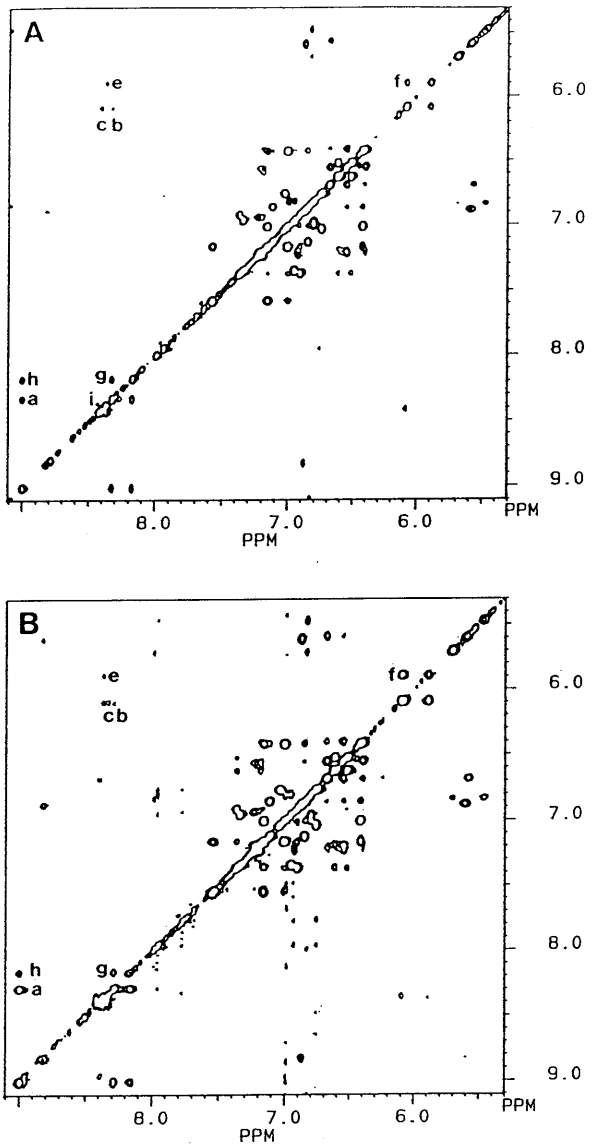

FIGURE 3: Aromatic–aromatic regions of the NOESY spectra of WT·MgAP$_5$A (A) at 34 °C and T23A·MgAP$_5$A (B) at 27 °C showing the assignment of H_2, H_8, and $H_{1'}$ of bound MgAP$_5$A (same samples used in Figure 2). The mixing time was 200 ms. The assignments are according to those in Yan et al. (1990b), with I and II designating the AMP moiety and the ATP moiety, respectively: the downfield peak at 9.00 ppm was assigned to H_2(I); peak a, exchange cross peak between H_2(I) and H_2(II); peak b, NOE cross peak between H_2(II) and $H_{1'}$(II); peak c, NOE cross peak between $H_{1'}$(II) and H_8(II); peak f, exchange cross peak between $H_{1'}$(I) and $H_{1'}$(II); peak e, NOE cross peak between H_8(I) and $H_{1'}$(I); peak g, exchange cross peak between H_2(II) and H_2(free); peak h, exchange cross peak between H_2(free) and H_2(I); peak i, exchange cross peak between H_8(I) and H_8(free). The exchange cross peak between H_8(I) and H_8(II) (labeled as peak d in Yan et al., 1990b) is obscured by the diagonal peaks. The assigned chemical shift values are listed in Table IV.

substrate protons, we also assigned the resonances of adenosine H_2, H_8 and ribose $H_{1'}$ of both AMP and ATP moieties (designated as I and II, respectively) of AK·MgAP$_5$A from the NOESY spectra shown in Figure 3, for both WT and T23A. The procedure used in the assignment is essentially the same as described in Yan et al. (1990a,b). The chemical shifts of these resonances are listed in Table IV. Some of these chemical shifts are slightly different from those reported in the previous studies (Yan et al., 1990a) due to different conditions used in the two experiments. Most noticeably, the order of H_8(I) and H_8(II) for WT has been reversed under the present condition. Comparison of the data in row 2 (WT·MgAP$_5$A) and row 3 (T23A·MgAP$_5$A) indicates that the resonance that differs the most between the two complexes

Table IV: Chemical Shifts (ppm) of Nucleotide Protons in AK·MgAP$_5$A Complexes[a]

complex	H_2	H_8	$H_{1'}$
WTAK·MgAP$_5$A[b]	8.98 (I), 8.32 (II)	8.47 (I), 8.40 (II)	5.93 (I), 6.07 (II)
WTAK·MgAP$_5$A	9.00 (I), 8.32 (II)	8.36 (I), 8.40 (II)	5.89 (I), 6.08 (II)
	8.17 (free)	8.43 (free)	6.07 (free)
T23A·MgAP$_5$A	9.00 (I), 8.29 (II)	8.37 (I), 8.33 (II)	5.89 (I), 6.09 (II)
	8.17 (free)	8.43 (free)	6.07 (free)

[a] Sites I and II have been assigned to the AMP and the MgATP moieties, respectively (Yan et al., 1990b). [b] From Yan et al. (1990a), at pH 7.8, 27 °C, and [Enz]/[MgAP$_5$A] = 1. The condition used in this work (optimal condition for our total assignment work) is slightly different: pH 7.5, 34 °C, and [Enz]/[MgAP$_5$A] = 0.5.

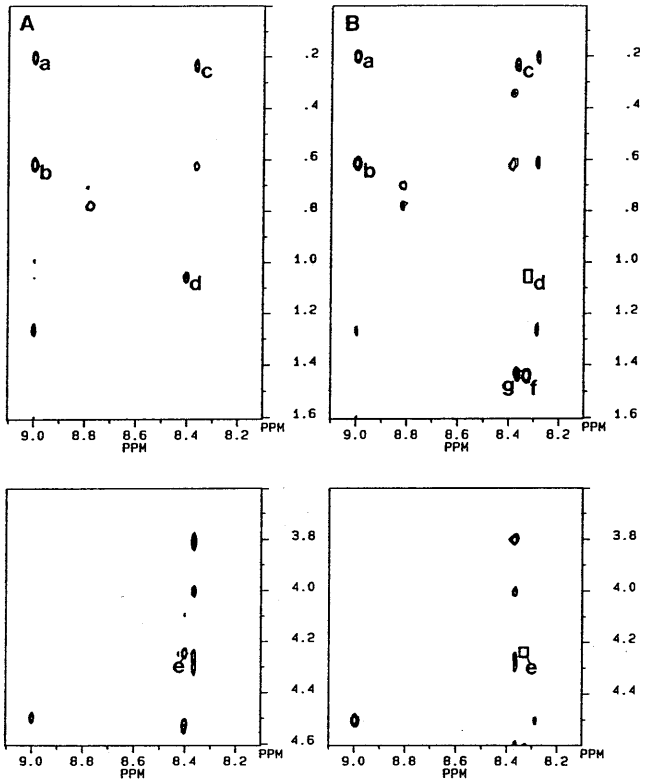

FIGURE 4: Partial aliphatic–aromatic NOESY spectra of WT·MgAP$_5$A (A) at 34 °C and T23A·MgAP$_5$A (B) at 27 °C (same samples used for Figure 2). Cross peaks d and e in (A) arise from NOE between the side chain γ,β-protons of Thr-23 and adenine H_8 of the ATP moiety. These two cross peaks disappear in B (as noted by squares) and are replaced by a new cross peak f arising from the methyl group of Ala-23 and the same adenine proton. Other assignments are explained in the text. The additional cross peaks at 8.29 ppm in (B) can also be observed in (A) when plotted at a lower level.

is H_8(II), the H_8 of the ATP moiety, which is also the proton experiencing NOE with the side chain protons of Thr-23 as described in the next section.

Identification of NOE between Thr-23 and the ATP Moiety in AK·MgAP$_5$A. Having assigned the resonances of Thr-23 and the bound MgAP$_5$A, we then looked for possible NOEs between them. Figure 4A shows part of the NOESY spectrum of WT·MgAP$_5$A complex, where intermolecular cross peaks between adenine H_2 or H_8 and side-chain protons of aliphatic amino acids can be observed. Among the cross peaks shown in this spectrum, cross peaks d and e can be assigned to NOE between the γ-CH_3 (1.05 ppm) and β-CH (4.24 ppm), respectively, of Thr-23, and the H_8 of the ATP moiety of AP$_5$A at 8.40 ppm. Cross peaks a and b have been assigned as intermolecular NOE cross peaks between H_2 of the AMP moiety and δ,δ'-protons of Leu-66, and cross peak c between

H_8 of the AMP moiety and δ-protons of Leu-43, based on sequential assignments (Byeon, unpublished results). These assignments support the locations of both the ATP moiety and the AMP moiety in the crystal structure of AKy·$MgAP_5A$ (Egner et al., 1987).

Further verification of the assignment of the NOE involving Thr-23 (cross peaks d and e) comes from the corresponding spectrum of T23A·$MgAP_5A$ (Figure 4B), where cross peaks a, b, and c (from the AMP moiety) remain unchanged but cross peaks d and e disappear. In addition, a new peak labeled as f appears at 1.44 ppm/8.33 ppm. On the basis of the chemical shift values listed in Tables III and IV, this new cross peak can be assigned to NOE between the methyl group of Ala-23 and the adenine H_8 of the ATP moiety in T23A·$MgAP_5A$. Another new peak, g, could arise from secondary NOE between β-CH_3 of Ala-23 and H_8 of the AMP moiety due to chemical exchange (AP_5A is symmetrical when dissociated from the enzyme). This was further verified by repeating the NOESY experiment with a shorter mixing time (120 instead of 200 ms), where only peak f is present (spectrum not shown). These results have clearly established that the side chain of Thr-23 (or Ala-23) is in proximity to the adenine ring of the ATP moiety of the AK1·$MgAP_5A$ complex in solution.

Rationale Behind Stereochemical Analysis. Without additional structural or functional analysis, the results from the previous sections taken together would suggest that the side chain of Thr-23 is "nonessential" for catalysis despite its proximity to the ATP moiety in the AK1·$MgAP_5A$ complex in solution. Such a dilemma can be rationalized in three possible ways: (i) despite the proximity, there is no direct interaction between the side chain of Thr-23 and ATP during catalysis; (ii) the catalytic role of the β-hydroxyl group of Thr-23 is substituted by a solvent molecule or a nearby functional group; and (iii) the side chain of Thr-23 does interact with ATP during catalysis, but this interaction contributes little to the energetics of catalysis.

Possibility i is not an unreasonable scenario if nothing else can be proven. Possibility ii is best tested by X-ray crystallography, but cocrystals of AK1 with a substrate or inhibitor have not yet been obtained despite the success with AKe and AKy. The striking similarity between the chemical shifts of WT·$MgAP_5A$ and T23A·$MgAP_5A$ as shown in Table II argues against any major conformational rearrangements; however, small local changes cannot be ruled out. Possibility iii can be tested by using substrate analogues. Since we have demonstrated recently that perturbations in the phosphorus stereospecificity can be used as an evidence for direct interaction between the mutated residue and the phosphate group (Jiang et al., 1991; Dahnke et al., 1992), this approach was used to further probe the functional role of Thr-23.

The Stereospecificity of T23A Is Significantly Perturbed. As shown in Scheme A1 of Figure 5, WT AK catalyzes the conversion of AMPS to (S_p)-ADPαS at the AMP site (Sheu & Frey, 1977; Jiang et al., 1991); the (S_p)-ADPαS formed is further converted to (S_p)-ATPαS at the MgATP site (Eckstein & Goody, 1976; Kalbitzer et al., 1983; Jiang et al., 1991). On the basis of quantitative analysis by ^{31}P NMR, the degree of stereospecificity at the AMP site is ca. 95% (i.e., ca. 5% of the R_p isomer of ADPαS is detectable), and that of the MgATP site is nearly 100% (the R_p isomer of ATPαS is undetectable). Thus in Scheme A2 of Figure 5 the formation of (R_p)-ADPαS from AMPS is indicated by small arrows, and the formation of (R_p)-ATPαS from (R_p)-ADPαS is crossed.

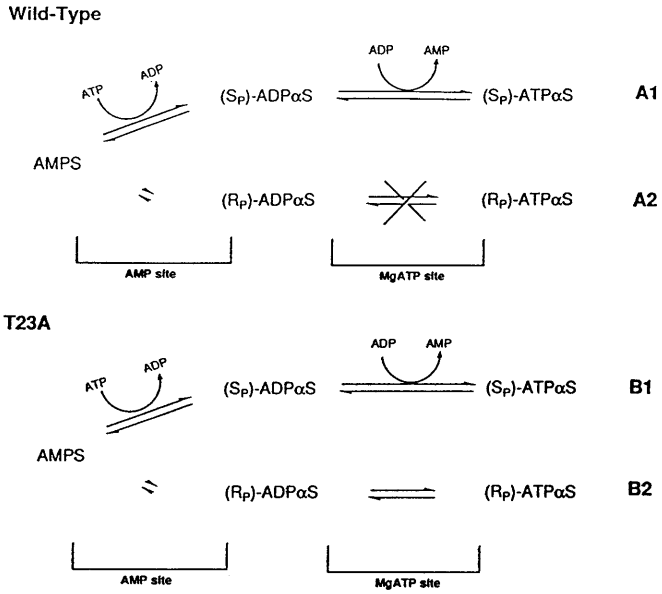

FIGURE 5: Schemes showing the stereospecificity of WT AK (A1 and A2) and T23A (B1 and B2).

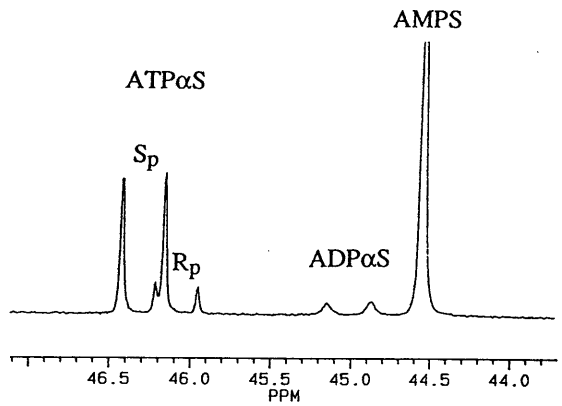

FIGURE 6: ^{31}P NMR analysis of the reaction of AMPS and MgATP catalyzed by T23A showing the presence of (R_p)-ATPαS. The starting reaction mixture (0.6 mL) consisted of 22 mM AMPS, 75 mM ATP, 45 mM $Mg(NO_3)_2$, and ca. 100 μg of T23A in 50 mM Tris buffer containing 50 mM KCl and 2.5 mM EDTA, pH 7.8. This spectrum was obtained in the absence of the deuterium lock. Data acquisition started right after the addition of T23A. For better resolution, samples were adjusted to pH 14 before the spectra were taken. Notice that the peak of AMPS is shifted upfield under this condition.

The ^{31}P NMR analysis of the reaction between AMPS and MgATP catalyzed by T23A is shown in Figure 6, which shows a noticeable difference from the result of WT (Jiang et al., 1991; Dahnke et al., 1992) in that there is a considerable amount of the R_p isomer of ATPαS (20% relative to S_p) in the product of T23A. This indicates a perturbation in the stereospecificity at the α-phosphate site of MgATP. The result, however, gives no indication as to the extent of perturbation in stereospecificity since the formation of (R_p)-ATPαS is limited by the availability of (R_p)-ADPαS, which is controlled by the stereospecificity of the AMP site. The result also gives no indication on the stereospecificity of the AMP site since (R_p)-ADPαS does not accumulate. Thus, this standard experiment only provides a qualitative indication on the relaxation of the stereospecificity at the P_α of ATP.

We therefore performed a more informative experiment: incubation of (R_p)-ADPαS and (S_p)-ADPαS with the enzyme. In the case of WT, this experiment produces exclusively (S_p)-ATPαS (and AMPS) at the expense of exclusively (S_p)-ADPαS (at both sites), leaving (R_p)-ADPαS unreacted (Dahnke et al., 1992). The results with T23A, as shown in

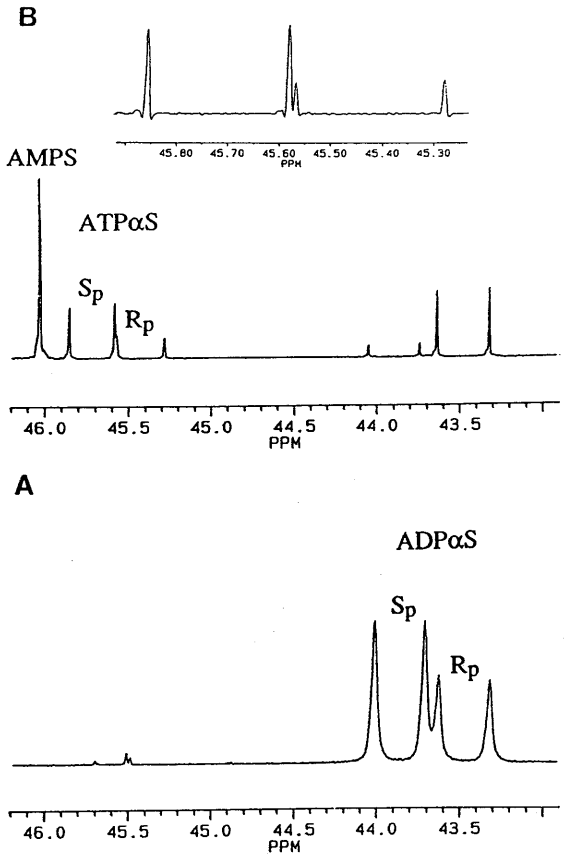

FIGURE 7: ^{31}P NMR analysis of the reaction of (R_p)-ADPαS and (S_p)-ADPαS catalyzed by T23A. (A) Starting substrates: (R_p + S_p)-ADPαS (S_p/R_p = 1.6); (B) reaction mixture, obtained at 30 min after the addition of T23A; the inset is resolution-enhanced analysis of the product (R_p + S_p)-ATPαS. Sample conditions were similar to those of Figure 6, except the replacement of ATP and AMPS with 22 mM ADPαS. For better resolution, samples were adjusted to pH 5.6 before the spectra were taken. Notice that the peak of AMPS is shifted downfield under this condition.

Figure 7, indicate that a mixture of ATPαS with R_p/S_p = 0.37:1 is formed (spectrum B). This ratio should represent the stereochemical perference at the α-phosphorus of the MgATP site since the enzyme has ample supply of either isomer of ADPαS (spectrum A; an excess of S_p isomer was used). Since the ratio of (R_p)-ATPαS/(S_p)-ATPαS is <0.02 (to the limit of detection) for WT (Dahnke et al., 1992), the ratio R_p/S_p has been enhanced by >20-fold at the P_α of the MgATP site for T23A. This conclusion is indicated in Schemes B1 and B2 of Figure 5.

Further Analysis of the ^{31}P NMR Results. Before the above results can be used to suggest that the side chain of Thr-23 interacts directly with the α-phosphate of ATP during the catalysis by WT AK, it is necessary to address two issues. The first issue is whether the perturbation in stereospecificity is specific to the MgATP site. If the stereospecificity at the AMP site is also relaxed, it may be argued that the stereospecificity is relaxed nonspecifically due to a perturbation in conformation or mechanism. This issue should be reflected in the R_p/S_p ratio of ADPαS *consumed* and thus can be addressed by quantitative analysis of the results of Figure 7. If the stereospecificity at the AMP site is strictly S_p, then the R_p isomer of ADPαS consumed should be equal to the R_p isomer of ATPαS formed, and AMPS should come exclusively from (S_p)-ADPαS. In other words, the ratio of [AMPS] + [(S_p)-ATPαS] + [(S_p)-ADPαS remaining] to [(R_p)-ATPαS] + [(R_p)-ADPαS remaining], defined as R, should be equal to the S_p/R_p ratio of the starting ADPαS (1.6). On the other hand, a relaxation in the stereospecificity of the AMP site should result in a larger R value since some of the (R_p)-ADPαS will be converted to AMPS at the AMP site, in addition to being converted to ATPαS at the MgATP site. The observed R value, on the basis of both peak height as well as integration of Figure 7B, is 1.8; after correcting for the fact that the stereospecificity at the AMP site is 95% instead of 100%, the observed R value becomes 1.65, which agrees with the predicted value of 1.6 within experimental errors. Thus, the stereospecificity at the AMP site of T23A is the same as that of WT.

The second issue is whether the perturbation in the stereospecificity (at the MgATP site) is a "preequilibrium effect", i.e., a kinetic effect. If the system is allowed to equilibrate indefinitely, R_p and S_p isomers should be present in approximately equal quantity since they should have similar free energies. Since equilibration between R_p and S_p isomers must occur *via* AMPS as an intermediate, it should occur for ADPαS before ATPαS. This is not the case as shown in Figure 7B. In addition, such equilibration should also make the observed R value deviate from the predicted value, which is not the case. Most importantly, the result is similar at an earlier stage of reaction (not shown).

The detailed ^{31}P NMR analyses described above allow us to interpret the stereochemical results of T23A: the side chain of Thr-23 interacts directly with the α-phosphate of ATP during the catalysis by WT AK, most likely *via* a hydrogen bonding.[3]

DISCUSSION

The results presented in this paper have led to the following conclusions: (i) Thr-23 is not very important for the function (except a small effect on K_i and K_m values) or the structure of AK1 from chicken muscle despite the fact that it is part of the P-loop and is conserved in all known AK sequences (Saraste et al., 1990). (ii) The side chain of Thr-23 is in proximity to the adenine ring of the ATP moiety in the AK1·$MgAP_5A$ complex in solution, which agrees with the crystal structure of the $MgAP_5A$ complex of the large variant of AK (Egner et al., 1987). (iii) The side chain of Thr-23 is likely to be involved in a hydrogen bonding interaction Thr–OH···O–P_α(ATP), which agrees with the refined crystal structure of AKe·$MgAP_5A$ published during the course of this work (Müller & Schulz, 1992). The significance and implication of these conclusions on the structure–function relationship of enzymes in general and AK in particular are discussed in the following sections.

The MgATP Site of Adenylate Kinase. Although the interactions of the AMP moiety and the transferring phosphoryl group of ATP with the active residues of AK have been well documented by site-specific mutagenesis (Tsai & Yan, 1991 and references therein; Dahnke et al., 1992; Okajima et al., 1991; Rose et al., 1991), all of the evidence for the MgATP site came from physical analyses of the AK·$MgAP_5A$ complexes by X-ray (Egner et al., 1987; Müller & Schulz, 1992) and NMR (Vetter et al., 1990, 1991). The AK used in these physical studies are the large variants, AKe and AKy. We therefore concluded (Tsai & Yan, 1991) that the MgATP site of muscle AK (AK1) requires additional evidence for the following reasons: (i) in the $MgAP_5A$ complexes of AKy and

[3] An alternative interpretation has been suggested by one of the reviewers: the perturbation in stereospecificity is caused by the steric bulk of sulfur relative to oxygen. In other words, the bulkier sulfur stays away from the bulkier Thr in WT, while no such constraint exists in T23A. Although such a factor cannot be completely ruled out, it is unlikely, in our view, that it will be solely responsible for the observed change in stereospecificity.

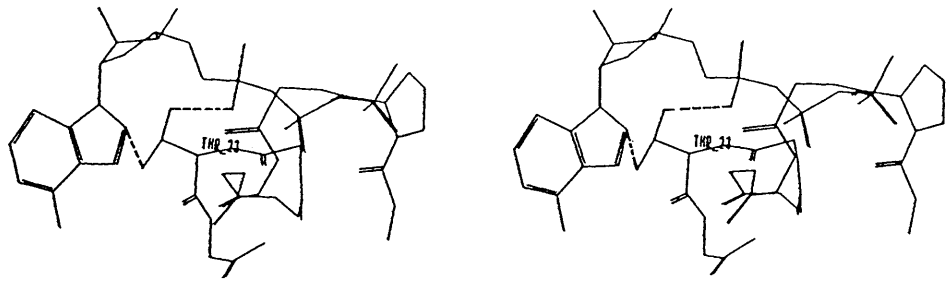

FIGURE 8: Stereostructure showing the H bond between the β-OH of Thr-23 and the α-phosphate of ATP, and the proximity between the γ-CH_3 of Thr-23 and the C_8 of the adenine ring (as indicated by dashed lines), constructed by docking ATP into the crystal structure of free AK1 from porcine muscle (Dreusicke et al., 1988). The conformation of ATP mimicks that of the ATP moiety in the crystal structure of AKe·$MgAP_5A$ (Müller & Schulz, 1992).

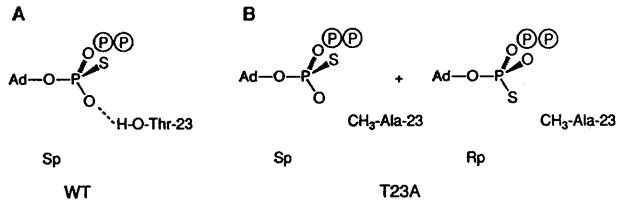

FIGURE 9: Structures illustrating the proposed stereospecific interactions with ATPαS at the active sites of WT and T23A, at the transition state.

AKe, the adenosine of the ATP moiety is surrounded by a 30-residue "insertion segment" which is absent in muscle AK (AK1); (ii) site-directed mutagenesis studies (of any type of AK) have not yet produced convincing evidence supporting that ATP actually binds to the site occupied by the ATP moiety of AP_5A; (iii) however slim, there is always a possibility of a difference between solution and crystal structures; (iv) recent chemical modification studies, performed in solution for chicken muscle AK, have produced results of MgATP site both in agreement (Cremo et al., 1992) and in contradiction (Salvucci et al., 1992) with that suggested from the crystal structures of AK·$MgAP_5A$.

Our NOE results suggest that the binding mode of $MgAP_5A$ with AK1 (small variant) in solution is likely to be similar to that in the crystal structure of AKy (large variant) as suggested by Egner et al. (1987). The "proximity", however, does not necessarily lead to direct binding interaction or a functional role. On the basis of the results of phosphorus stereochemistry, the hydroxyl group of Thr-23 appears to be interacting directly, most likely via a hydrogen bond, with the phosphoryl group at the α-position of the *substrate* ATP. Since such an interaction has also been suggested for the ATP moiety of the *inhibitor* AP_5A in the crystal structure of AKe·$MgAP_5A$ (Müller & Schulz, 1992), our structural and functional results on the binding mode of MgATP in solution are consistent with that of the ATP moiety of $MgAP_5A$ in the crystal structure. The relationship between the side chain of Thr-23 and MgATP on the basis of our NOE and stereochemistry results is depicted in Figure 8.

The fact that sulfur is a poorer H-bond acceptor than oxygen, and that the R_p isomer increases upon removal of the hydrogen bond, suggests that in the WT·(S_p)-ATPαS complex (at the transition state) the oxygen instead of the sulfur is involved in the hydrogen bond with Thr-23, as shown in Figure 9A. Removal of the OH group of Thr-23 allows the R_p isomer to bind, as shown in Figure 9B. This would predict that with the natural ATP substrate the *pro-R* oxygen is involved in the H-bonding with the Thr-23. Such analysis is based on the assumption that the stereospecificity is controlled mainly by this particular hydrogen bond; however, the conclusion is consistent with the crystal structure of AKe·$MgAP_5A$ (Müller & Schulz, 1992).

Implications on the Structure–Function Relationship of Enzymes. How can Thr-23 participate in catalysis by interacting with the α-phosphate of ATP but not contribute to the energetics of catalysis? We believe that the interaction does contribute ca. 1.8 kcal/mol to catalysis on the basis of the 20-fold enhancement in the formation of the R_p isomer of ATPαS. The fact that this does not lead to a significant binding effect *specific to MgATP* and/or a noticeable decrease in k_{cat} in the T23A mutant could be caused by two possible reasons: (i) the energetic contribution by Thr-23 *via* interacting with the phosphate group is partially or totally canceled by other unfavorable interactions involving this residue; and (ii) the mutant T23A does not behave exactly like WT minus the β-hydroxyl group of Thr-23; small changes occur in various microscopic steps.

The results in this paper demonstrate the importance in rigorous structure-function analyses of site-specific mutants. Without knowing the proximity between Thr-23 and ATP, we would have concluded that Thr-23 does not participate in catalysis. Had the phosphorus stereochemistry experiments not been performed, we would have concluded that the side chain of Thr-23 is not involved in catalysis despite its proximity to ATP. In the past we have emphasized that the "proximity" between a side chain and a substrate cannot be equated with direct interaction or catalytic contribution (Tsai & Yan, 1991). The present example sends another message in the structure-function study of enzymes: lack of significant perturbation in kinetic parameters of a mutant may not necessarily mean that the mutated residue does not participate directly in catalysis. In other words, *site-directed mutagenesis could fail to identify functional (or structural) residues unless rigorous chemical and physical characterizations can be performed.*

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