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Mechanism of Adenylate Kinase. Does Adenosine 5′-Triphosphate Bind to the Adenosine 5′-Monophosphate Site?†

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ABSTRACT: Although the substrate binding properties of adenylate kinase (AK) have been studied extensively by various biochemical and biophysical techniques, it remains controversial whether uncomplexed adenosine 5′-triphosphate (ATP) binds to the adenosine 5′-monophosphate (AMP) site of AK. We present two sets of experiments which argue against binding of ATP to the AMP site. (a) ^{31}P nuclear magnetic resonance titration of ATP with AK indicated a 1:1 stoichiometry on the basis of changes in coupling constants and line widths. This ruled out binding of ATP to both sites. (b) ATP and MgATP were found to behave similarly by protecting AK from spontaneous inactivation while AMP showed only a small degree of protection. Such inactivation could also be protected or reversed by dithioerythritol and is most likely due to oxidation of sulfhydryl groups, one of which (cysteine-25) is located near the MgATP site. The results support binding of ATP to the MgATP site predominantly, instead of the AMP site, in the absence of Mg^{2+}.

Adenylate kinase (AK)[1] (Noda, 1973) catalyzes the reaction

$$MgATP + AMP \rightleftharpoons MgADP + ADP$$

Although the three-dimensional structure of AK from porcine muscle has been solved by X-ray crystallography (Schulz et al., 1974; Pai et al., 1977), the substrate binding sites have not been well-defined in the crystal structure. In the past decade, a number of reports have addressed the location and the binding properties of the substrate binding sites, which are summarized below. *Unless otherwise specified, the discussion in this paper focuses on AK from porcine muscle and rabbit muscle*, which are 95% homologous (Kuby et al., 1984). All of our work used AK from porcine muscle.

The active site of AK consists of a "MgATP site" and an "AMP site" (Rhoads & Lowenstein, 1968; Noda, 1973). When MgATP and AMP are converted to MgADP and ADP, respectively, the MgATP site should bind MgADP, and the AMP site should bind ADP. The enzyme also binds Ap_5A and $MgAp_5A$ tightly (Price et al., 1973; Nageswara Rao & Cohn, 1977; Yazawa & Noda, 1976). Kupriyanov et al (1986) have shown that AK also catalyzes the formation (very slowly) of adenosine 5′-tetraphosphate, presumably from MgATP and ADP.

The location of the two sites proposed by Pai et al. (1977) based on the crystal structure of an AK–salicylate complex was inconsistent with the NMR studies of Smith and Mildvan (1982), as well as the elegant work of Hamada et al. (1979), who identified a "MgATP binding fragment" (residues 1–44) and an "AMP binding fragment" (residues 171–193).[2] Further ^1H NMR studies on the MgATP binding fragment and the intact AK have revealed the detailed interactions between metal–ATP and AK (Fry et al., 1985; Mildvan & Fry, 1987).

In terms of functional properties, an important difference between the two sites is that the AMP site binds only uncomplexed adenine nucleotides while the MgATP site binds complexed as well as uncomplexed nucleotides, as suggested by kinetic studies (Rhoads & Lowenstein, 1968), ^{31}P NMR experiments (Nageswara Rao et al., 1978), and the binding studies of the two peptide fragments (Hamada et al., 1979). However, the following important question remains: *When AK is mixed with a stoichiometric amount or excess of an uncomplexed nucleotide (AMP, ADP, or ATP) as in many biophysical and biochemical studies, does the uncomplexed nucleotide go to a preferred site, or both sites*? The problem is particularly intriguing when we consider that AK also binds uncomplexed Ap_5A tightly as mentioned above.

In the case of ADP, a stoichiometry of 2.0 has been obtained in the absence of Mg^{2+} (and 1.0 in the presence of Mg^{2+}) (Hamada et al., 1979), which suggests that uncomplexed ADP can bind to both sites. However, AMP seems to bind to the AMP site preferentially. A stoichiometry of 1 has been reported for ϵAMP binding to AK from rabbit muscle and calf muscle (Hamada et al., 1979). Rhoads and Lowenstein (1968) reported that AMP is a competitive inhibitor of MgATP with K_i = 50 mM, which is much larger than K_m and K_d values of ca. 10^{-4} M for MgATP (Noda, 1973; Hamada et al., 1979). Hamada et al. (1979) also showed that the MgATP binding fragment of rabbit muscle AK binds ϵAMP only weakly ($K_d \geq 5$ mM, compared to 0.23 mM for binding of ϵAMP to the AMP binding fragment).

The problem with ATP is, however, still controversial and is the focus of this report. For yeast AK, the stoichiometry

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; AK, adenylate kinase; AMP, adenosine 5′-monophosphate; Ap_5A, P^1,P^5-di(adenosine-5′) pentaphosphate; ATP, adenosine 5′-triphosphate; DTE, dithioerythritol; ϵAMP, 1,N^6-ethenoadenosine 5′-monophosphate; ϵADP, 1,N^6-ethenoadenosine 5′-diphosphate; ϵATP, 1,N^6-ethenoadenosine 5′-triphosphate; CD, circular dichroism; EDTA, ethylenediaminetetraacetate; HPLC, high-pressure liquid chromatography; MES, 2-(N-morpholino)ethanesulfonic acid; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; NMR, nuclear magnetic resonance; SDS, sodium dodecyl sulfate; UV, ultraviolet.

[2] The binding studies reported in Hamada et al. (1979) were performed with etheno analogues of adenine nucleotides (ϵAMP, ϵADP, and ϵATP). In the discussion of this paper, we compare these data directly with other reports using AMP, ADP, and ATP.

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of ATP binding has been reported as 0.92 by Ito et al. (1980) and 2.3 by Su and Russell (1967). For muscle AK, the equilibrium binding experiments of rabbit muscle AK revealed a stoichiometry of 1.8 for both ATP and MgATP (Kuby et al., 1962), whereas a stoichiometry of 1 was concluded from proton relaxation rate studies of porcine muscle AK (Price et al., 1973) and from the fluorescent quenching and UV difference spectral studies of rabbit and calf muscle AK (Hamada et al., 1979). In the ^{31}P NMR study of Nageswara Rao et al. (1978), two signals were observed for the P_β of ATP when the Mg^{2+}/ATP/AK (porcine) ratio was 5.8/2.5/2.7 (mM). The lower field signal (−19 ppm) was assigned to E·ATP·Mg, whereas the other signal at −21.5 ppm was attributed to the binding of uncomplexed ATP to the AMP site, forming a quaternary complex, AK·ATP·Mg·ATP. Such a complex has also been suggested by Berghauser (1975) for the AK from porcine heart. The observation that the AMP binding fragment binds ϵATP very weakly (Hamada et al., 1979) does not necessarily apply to the intact AK, since in the intact AK the AMP site is extended to (or overlaps with) the MgATP site and is more likely to bind ATP.

Although uncomplexed ATP is not a natural substrate of AK, the issue of whether it binds to the AMP site is important in understanding the active-site structure of AK and in properly interpreting the results of biochemical and physical studies. For example, the binding properties of uncomplexed ATP to AK have been characterized by ^1H NMR (McDonald et al., 1975), ^{31}P NMR (Nageswara et al., 1978), ^{17}O NMR (Wisner et al., 1985), UV difference spectroscopy (Hamada et al., 1979), and CD difference spectroscopy (Yazawa & Noda, 1976). These results would require reinterpretation if ATP can bind to both sites, or even to the AMP site exclusively, in the absence of Mg^{2+}.

In this paper, we present two pieces of evidence which support that ATP does not bind to the AMP site. (a) ^{31}P NMR titration of ATP with AK indicates 1:1 stoichiometry, which excludes the possibility of ATP binding to both sites. (b) Both ATP and MgATP show a property similar to DTE in protecting AK from spontaneous inactivation, while AMP shows only a small degree of protection. This suggests that ATP binds to the same site as MgATP.

Materials and Methods

Materials. General biochemicals and the enzymes used in the assay of AK were purchased from Sigma. Ion-exchange resins were obtained from Pharmacia or Sigma. Chelex-100 was purchased from Bio-Rad. All other reagents were reagent grade.

Purification and Assay of AK. Adenylate kinase was purified from porcine muscle according to the procedure described by Schirmer et al. (1970), with the following modifications. Affinity elution with 5 mM ATP and 5 mM AMP in 0.155 M imidazole buffer, pH 7.0, instead of gradient elution was used in the phosphocellulose column step (step 7). Sephadex G-50, instead of Sephadex G-75, was used in gel filtration (step 8). The crystallization step was replaced by ammonium sulfate precipitation (90%). The enzyme was then centrifuged down, the supernatant discarded, and the pellet covered with a holding buffer containing 3.2 M $(NH_4)_2SO_4$, 1 mM EDTA, and 5 mM DTE, pH 6.0, and stored at 4 °C. Homogeneity of the purified AK was examined by SDS gel electrophoresis and hydrophobic interaction HPLC on a poly(propylaspartamide) column (4.6 × 200 mm; Nest Group, Southboro, MA) with elution by a linear gradient from 2.0 to 0 M ammonium sulfate in 0.1 M potassium phosphate buffer, pH 6.5. A single peak was observed in the HPLC profile for purified AK (Wisner, 1987). An isocratic elution system with 1.0 M ammonium sulfate and 0.1 M potassium phosphate, pH 6.4, gave similar results. Whenever necessary, AK was further purified by HPLC using these conditions, with full recovery of activity. The enzyme used in NMR experiments was recovered by precipitation with ammonium sulfate (90%) and repeating the gel filtration step and the final precipitation.

The activity of the enzyme was assayed at 30 °C by coupled reactions with pyruvate kinase and lactate dehydrogenase. The concentration of AK was measured spectrophotometrically at 280 nm using an extinction coefficient of 0.54 for a 0.1% solution (Schirmer et al., 1970). The specific activity of the enzyme used in the NMR experiment was ca. 1800 units/mg.

^{31}P NMR Experiments. The enzyme (ca. 100 mg) from the storing pellet was redissolved in a small volume of 0.07 M triethanolamine hydrochloride buffer containing 65 mM KCl and 1.5 mM DTE, pH 7.8. The solution was then dialyzed in a Spectrapor dialyzing tube (molecular weight cutoff ca. 3500) against 500 mL of 15 mM EDTA, pH 7.8, 2 times, followed by three 300-mL dialyses against the same triethanolamine buffer described above. The solution was then concentrated in a Minicon microsolute concentrator to ca. 3.0–3.5 mM.

The nucleotide samples were first converted to sodium salts by passing through a SP-Sephadex C-25 column. The sample was then passed through a small column of Chelex-100 to remove trace contaminating metal ions. After lyophilization, the nucleotide was redissolved in D_2O, titrated to pD 7.8 (direct reading) with puratronic grade NaOH, quantified by UV absorbance at 259 nm, and kept frozen as a stock solution.

^{31}P NMR experiments were conducted at 81.0 MHz on a Bruker WP-200 NMR spectrometer, with deuterium lock, at 10 °C in all cases. The chemical shifts were referenced to external 85% H_3PO_4 (at 30 °C), with a plus sign indicating a downfield shift.

Spontaneous Inactivation of AK. A concentrated enzyme solution (ca. 1 mM) was dialyzed against 75 mM MES buffer containing 65 mM KCl and 1.5 mM DTE, pH 5.9, and kept at 4 °C. Two stock solutions were prepared and incubated at 25 °C: solution A contained 75 mM MES and 65 mM KCl, pH 5.9 (without DTE), while solution B contained 75 mM MES, 65 mM KCl, and 20 mM DTE or the desired adenine nucleotide, pH 5.9. In the study with MgATP, 3 equiv of Mg^{2+} (relative to ATP) was used. To start an experiment, the AK solution was diluted by a factor of ca. 10^4 into solution A to reach a concentration of 2 μg/mL. In the control experiment (without DTE or nucleotide), this AK solution was incubated at 25 °C, and its activity was monitored at time intervals by withdrawing an aliquot to assay the activity. In other experiments, the dilute AK solution was mixed with one-ninth of solution B (so that the final concentration of DTE or nucleotide was 2.0 mM) and the activity monitored accordingly. pH 5.9 was used since at this pH the dilute AK solution was more stable and still showed a specific activity of 1100 units/mg.

Results and Discussion

Stoichiometry of [ATP]/[AK] by ^{31}P NMR. Binding of free ATP to AK causes little change in ^{31}P chemical shifts (Nageswara Rao et al., 1978), which makes quantitation difficult. However, the small changes in $J_{\alpha\beta}$ and $J_{\beta\gamma}$, as well as the small increase in the line width of ^{31}P NMR signals (ΔP), can be used to determine the stoichiometry of binding. We measured the ^{31}P NMR spectra of ATP in the presence of varying concentrations of AK from porcine muscle. Figure

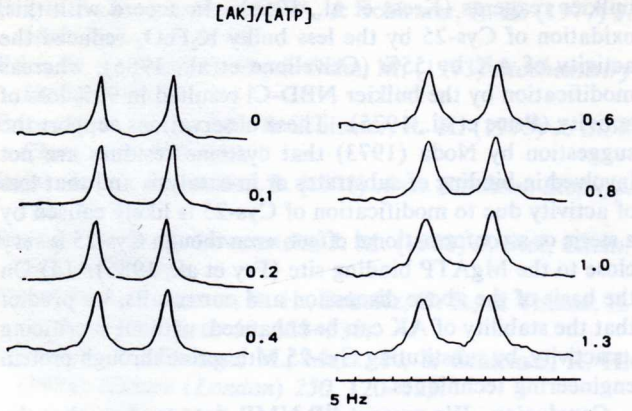

FIGURE 1: ^{31}P NMR signals of the P_α of ATP free and bound to varying concentrations of AK at pH 7.8 and 10 °C. Spectral parameters: spectral width, 5000 Hz; 16K data points; acquisition time, 1.6 s; 60° pulse; broad-band ^1H decoupling; line broadening, 2 Hz; 4000–8000 scans. The starting solution contained 2.3 mM ATP, 70 mM triethanolamine hydrochloride, 65 mM KCl, 1.5 mM DTE, and 20% D_2O. A solution of 3.0 mM AK was added to the starting solution to reach the molar ratios of [AK]/[ATP] shown in the figure. The small chemical shift changes between different spectra are within experimental error.

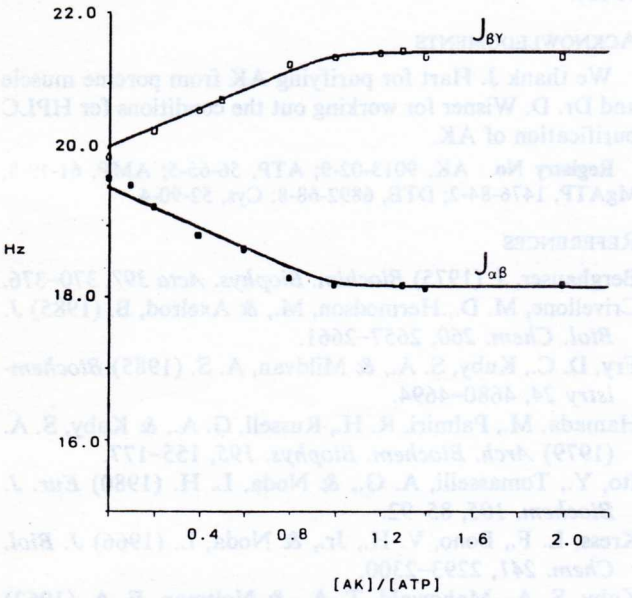

FIGURE 2: ^{31}P–^{31}P spin–spin coupling constants $J_{\alpha\beta}$ and $J_{\beta\gamma}$ as a function of [AK]/[ATP]. The sample and spectral conditions are described in Figure 1. The digital resolution is 0.61 Hz/point.

1 shows the expanded P_α signals from part of the titration experiment. The plots of $J_{\alpha\beta}$ and $J_{\beta\gamma}$ in Figure 2, and that of ΔP_α and ΔP_γ in Figure 3, show linear changes from [AK]/[ATP] = 0 to [AK]/[ATP] = 1. The actual stoichiometries determined from nonlinear least-squares fitting of the data, assuming a binding constant $K_a = 3 \times 10^4$ M^{-1} (Price et al., 1973), are 1.04 ± 0.17, 0.98 ± 0.06, 1.00 ± 0.02, and 1.00 ± 0.03 based on $J_{\alpha\beta}$, $J_{\beta\gamma}$, ΔP_α, and ΔP_γ, respectively.

Protection of AK Inactivation by ATP, MgATP, and DTE. The 1:1 stoichiometry obtained above argues against binding of ATP to both sites but does not completely rule out the possibility of ATP binding to the AMP site preferentially in the AK·ATP binary complex. Since MgATP binds to the MgATP site specifically and AMP binds to the AMP site preferentially, unequivocal evidence to support binding of ATP to the MgATP site instead of the AMP site of native AK would be to find a particular property which is common to ATP and MgATP, but is different for AMP. Such a property

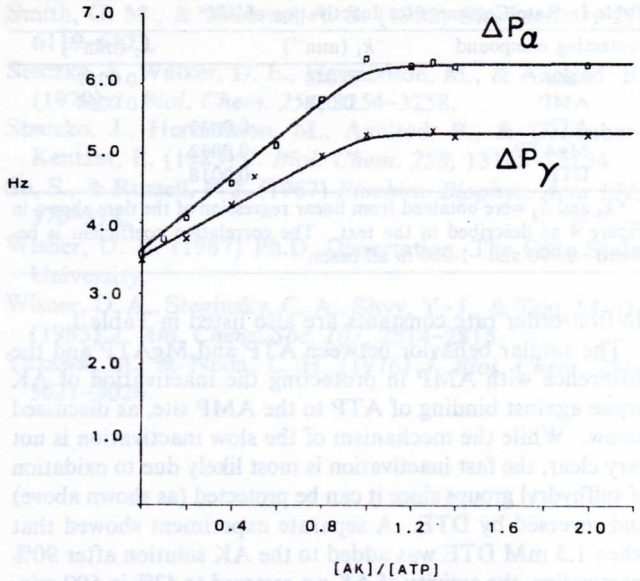

FIGURE 3: Plots of apparent line widths of ^{31}P NMR signals (ΔP) as a function of [AK]/[ATP] for P_α and P_γ. The sample and spectral conditions are described in Figure 1. The line widths include 2 Hz of artificial line broadening and are measured at the half-height and as the average of the two peaks.

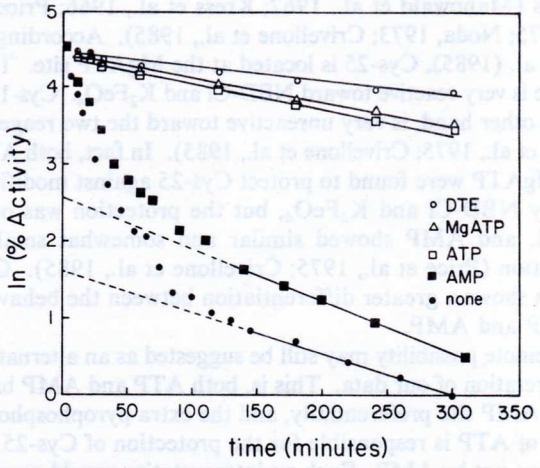

FIGURE 4: Plots of ln (percent activity) as a function of time for AK under various conditions, as described under Materials and Methods. The activity of AK at the first point of each experiment (1 min) is set as 100%.

has not been reported previously.

We have found that both ATP and MgATP show almost full protection of AK against spontaneous inactivation, while AMP shows only a small degree of protection. Although AK is stable in a concentrated solution, or in the presence of 1 mg/mL bovine serum albumin, a dilute solution of AK (0.1 μM) in the absence of a reducing agent quickly lost 90% of its activity in the first 40 min, followed by a slower decrease of activity. The inactivation was slowed down significantly by 2.0 mM DTE, ATP, or MgATP, but only slightly by AMP.

Figure 4 shows the semilogarithmic plots of percent activity vs. time. In the control experiment and in the presence of 2 mM AMP, the inactivation is clearly biphasic. The pseudo-first-order rate constant of the "slow" phase (k_2) was calculated from the linear regression of the linear portion (last five points) of the plot and is listed in Table I. The pseudo-first-order rate constant of the "fast" phase (k_1) was calculated from the first five points of the plot after subtraction of the contribution of the slow process. In the presence of 2 mM DTE, MgATP, or ATP, only a single phase is clearly identifiable, which may be due to incomplete protection of either process. The pseu-

Table I: Rate Constants for Inactivation of AK[a]

protecting compound	k_1 (min^{-1})	k_2 (min^{-1})
none	0.046	0.0057
AMP	0.032	0.0064
ATP	0.0033	
MgATP	0.0032	
DTE	0.0018	

[a] k_1 and k_2 were obtained from linear regression of the data shown in Figure 4 as described in the text. The correlation coefficient is between −0.990 and −1.000 in all cases.

do-first-order rate constants are also listed in Table I.

The similar behavior between ATP and MgATP and the difference with AMP in protecting the inactivation of AK argue against binding of ATP to the AMP site, as discussed below. While the mechanism of the slow inactivation is not very clear, the fast inactivation is most likely due to oxidation of sulfhydryl groups since it can be protected (as shown above) and reversed by DTE. A separate experiment showed that when 1.5 mM DTE was added to the AK solution after 90% inactivation, the activity of AK wa restored to 43% in 500 min, with a pseudo-first-order rate constant of 0.012 min^{-1}. Both porcine muscle AK and rabbit muscle AK have only two cysteine residues, Cys-25 and Cys-187 (Kuby et al., 1984), which have been the subject of extensive chemical modification studies (Mahowald et al., 1962; Kress et al., 1966; Price et al., 1975; Noda, 1973; Crivellone et al., 1985). According to Fry et al. (1985), Cys-25 is located at the MgATP site. This residue is very reactive toward NBD-Cl and K_2FeO_4. Cys-187, on the other hand, is very unreactive toward the two reagents (Price et al., 1975; Crivellone et al., 1985). In fact, both ATP and MgATP were found to protect Cys-25 against modification by NBD-Cl and K_2FeO_4, but the protection was only partial, and AMP showed similar and somewhat smaller protection (Price et al., 1975; Crivellone et al., 1985). Our system shows a greater differentiation between the behavior of ATP and AMP.

A remote possibility may still be suggested as an alternative interpretation of our data. This is, both ATP and AMP bind to the AMP site preferentially, and the extra pyrophosphoryl group of ATP is responsible for the protection of Cys-25 by ATP but not by AMP. Such an interpretation would require that Cys-25 be located in between the two sites (i.e., near the central phosphate group of Ap_5A in the AK·Ap_5A complex) and is protected when ATP is bound to either site. This possibility can be ruled out since Fry et al. (1985) have shown that Cys-25 is located near the ribose moiety of bound metal–ATP, ca. 5 Å away from the α-phosphate, and even further removed from the AMP site.

We further discuss four points which are not directly related to the conclusion of this paper but are pertinent to the structure and function of AK in general. (a) As described in the introduction, AMP binds to the AMP site preferentially but may also bind to the MgATP site with low affinity. This may account for the small degree of protection by AMP against spontaneous inactivation and against chemical modifications by NBD-Cl and K_2FeO_4. (b) The inability of ATP to effectively protect against chemical modifications could be because both NBD-Cl and K_2FeO_4 are somewhat active-site-directed and compete with ATP for the active site. The former could be due to its aromatic ring and the latter due to its being an analogue of orthophosphate (Lee & Banisek, 1976, 1978; Rajababu & Axelrod, 1978; Steczko et al., 1979, 1983). (c) The AK modified with mercurial reagents at both Cys-25 and Cys-187 exhibited unchanged K_m's, while the catalytic activity was reduced by 55% for a less bulky reagent and by 90% for bulkier reagents (Kress et al., 1966). In accord with this, oxidation of Cys-25 by the less bulky K_2FeO_4 reduced the activity of AK by 55% (Crivellone et al., 1985), whereas modification by the bulkier NBD-Cl resulted in 99% loss of activity (Price et al., 1975). These observations support the suggestion by Noda (1973) that cysteine residues are not involved in binding of substrates or in catalysis and that loss of activity due to modification of Cys-25 is likely caused by a steric or a conformational effect, even though Cys-25 is very close to the MgATP binding site (Fry et al., 1985). (d) On the basis of the above discussion and our results, we predict that the stability of AK can be enhanced, without sacrificing its activity, by substituting Cys-25 with serine through protein engineering techniques.

Conclusion. We present ^{31}P NMR data to show that the stoichiometry of ATP binding to AK from porcine muscle is 1:1. ATP and MgATP behave indistinguishably in protecting AK from inactivation due to oxidation of sulfhydryl groups, whereas AMP shows only a small degree of protection. The results support binding of uncomplexed ATP to the MgATP site, not the AMP site. The results also justify the assumption of 1:1 binding of ATP to the MgATP site of AK in the interpretation of our recent ^{17}O NMR studies (Wisner et al., 1985).

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Registry No. AK, 9013-02-9; ATP, 56-65-5; AMP, 61-19-8; MgATP, 1476-84-2; DTE, 6892-68-8; Cys, 52-90-4.

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