Nuclear Magnetic Resonance Studies of the Nucleotide Binding Sites of Porcine Adenylate Kinase†

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ABSTRACT: The α,β,γ-tridentate complex of CrATP, a paramagnetic competitive inhibitor of porcine adenylate kinase, increases the longitudinal $[1/(fT_{1p})]$ and transverse $[1/(fT_{2p})]$ relaxation rates of a resonance of the enzyme previously assigned by McDonald et al. [McDonald, G. G., Cohn, M., & Noda, L. (1975) *J. Biol. Chem. 250*, 6947–6954] to the C2 proton of histidine-36. These paramagnetic effects are diminished upon the addition of the substrate MgATP by an amount consistent with the simple displacement of CrATP. The $1/(fT_{2p})$ value sets a lower limit of 400 s^{-1} on the rate constant for dissociation of CrATP from the enzyme. The $1/(fT_{1p})$ value at 250 MHz and the correlation time for water protons in the same complex are used to calculate a distance of 12.9 ± 1.0 Å from Cr(III) to the C2 proton of histidine-36. A primary, negative nuclear Overhauser effect is detected on the adenine H2 resonance of enzyme-bound MgATP upon preirradiation of the C2 proton of histidine-36, indicating that these protons are ≲5 Å apart. These distances and negative intramolecular Overhauser effects from the ribose protons to adenine H8 of MgATP indicate an extended structure for bound MgATP with an anti conformation about the glycosidic bond. These findings require a different orientation or location of the bound metal–ATP substrate from that proposed based on X-ray studies of the binding of salicylate [Pai, E. F., Sachsenheimer, W., & Schirmer, R. H. (1977) *J. Mol. Biol. 114*, 37–45]. Other nuclear Overhauser effects from resonances of the protein at 1.8 and 0.9 ppm to both adenine H2 and ribose H1′ of bound MgATP indicate the proximity to the substrate of at least one Arg Cβ proton (at 1.8 ppm), Cγ proton (at 1.7 ppm), Lys Cδ proton (at 1.7 ppm), or Leu Cβ proton (at 1.6 ppm) and one or more Leu, Ile, or Val methyl groups (at 0.9 ppm). Entirely different Overhauser effects are observed from the enzyme to the adenine protons of AMP consistent with a distinct site for the other substrate.

Adenylate kinase catalyzes the phosphorylation of AMP by MgATP (Colowick & Kalckar, 1943; Noda, 1973; Nageswara Rao et al., 1978). The enzyme is widely distributed in nature and has been purified to homogeneity from a number of sources (Noda, 1973). Adenylate kinases isolated from muscle tissue are monomeric globular proteins having molecular weights near 21 000 (Noda, 1973). The primary structures of several muscle adenylate kinases are known, and an X-ray structure of the porcine muscle enzyme is available (Schulz et al., 1974; Sachsenheimer & Schulz, 1977).

Several studies have indicated that the two nucleotide binding sites are not functionally equivalent; one, the "ATP site", binds MgADP or MgATP, and the other, the "AMP site", binds ADP or AMP (Rhoads & Lowenstein, 1968). The differences in the binding properties of the two sites were delineated further by direct observation of the phosphorus resonances of bound nucleotides by ^{31}P NMR which demonstrated that the AMP site binds only uncomplexed nucleotides (AMP, ADP, or ATP), while the ATP site binds either ATP or ADP with or without the coordinated metal ion (Nageswara Rao et al., 1978). The structural nonequivalence of the binding sites was demonstrated most elegantly when Hamada et al. (1979) isolated two nonoverlapping peptide fragments of rabbit adenylate kinase, each containing only one nucleotide binding site, that mimicked the nucleotide binding sites of the native enzyme both qualitatively in specificity and quantitatively in affinity for fluorescent analogues of the nucleotides. the MgATP site was thus localized in residues 1–44 and the AMP site, in residues 171–193.

Nuclear magnetic techniques such as the measurement of intermolecular nuclear Overhauser effects (NOE's) (Bothner-By & Gassend, 1973; James, 1976; Vasak et al., 1979) and paramagnetic probe studies (Mildvan & Gupta, 1978; Mildvan et al., 1980) have been found to be valuable tools for studying enzyme–substrate interactions. The low molecular weight of porcine adenylate kinase relative to the other known kinases renders it particularly amenable to study by ^1H NMR techniques. We have therefore employed the nuclear Overhauser effect and the paramagnetic probe α,β,γ-tridentate $Cr^{III}ATP$ in an effort to gain more information about the location of the MgATP binding site of porcine adenylate kinase.

Materials and Methods

Porcine adenylate kinase was purchased from Sigma. The commercial preparation was found to be about 60% pure by polyacrylamide gel electrophoresis. The major contaminant was removed by gel filtration chromatography on Sephadex G-50 (1.5 × 100 cm) equilibrated with 50 mM Tris-HCl,[1] pH 7.5. The contaminant, which had a molecular weight of ~60 000, was eluted as a sharp band in the void volume, and adenylate kinase [M_r 21 700 (Noda, 1973)] appeared in the included volume. The concentration of the purified enzyme was determined by its absorbance at 280 nm [$\epsilon_{1mg/mL} = 0.52$ (Noda, 1973)]. Adenylate kinase activity was measured spectrophotometrically by the pyruvate kinase–lactate dehydrogenase coupled assay as described by Price et al. (1973).

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[1] Abbreviations: Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); DSS, sodium 4,4-dimethyl-4-silapentanesulfonate.

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Table 1: Relaxation Rates of Histidine and Water Protons in the CrATP–Adenylate Kinase Complex

resonance	additions	ν_I (MHz)	$1/T_1$ (s^{-1})	$1/T_{1p}$ (s^{-1})	$1/T_2$ (s^{-1})	$1/T_{2p}$ (s^{-1})	$1/(fT_{1p})$ (s^{-1})	$1/(fT_{2p})$ (s^{-1})	τ_c (s)	r (Å)
His-36 C2 H		250	0.741 ± 0.005		23 ± 3					
	+22 μM CrATP	250	1.493 ± 0.15	0.752 ± 0.15	36 ± 5	13 ± 6	24.6 ± 5.0	425 ± 196		12.9 ± 1.0
	+1.25 mM MgATP	250	1.17 ± 0.2	0.43 ± 0.2	29 ± 5	6 ± 6				
H_2O		250	0.116 ± 0.006		0.24 ± 0.01					
		360	0.119 ± 0.009		0.60 ± 0.02					
	+110 μM CrATP	250	0.230 ± 0.01	0.114 ± 0.01	0.33 ± 0.01	0.09 ± 0.01 [c]			5.5×10^{-10} [a]	
		360	0.198 ± 0.02	0.079 ± 0.02	0.66 ± 0.01	0.06 ± 0.02 [c]			8.0×10^{-10} [b]	

[a] Assumes no frequency dependence of τ_c. [b] Assumes maximum frequency dependence of τ_c. [c] Within experimental error $1/T_{2p} = 1/T_{1p}$ for water protons.

The purified enzyme had a specific activity of 1800 units/mg.

The α,β,γ-tridentate complex CrATP was prepared as described by Dunaway-Mariano & Cleland (1980a). First, β,γ-bidentate CrATP was prepared by incubation of 10 mM $CrCl_3$ with 10 mM Na_2ATP at pH 5.7 for 30 min at room temperature. After chromatographic separation on Dowex 1 (Cl^-), the β,γ-bidentate CrATP was allowed to stand for 24 h at pH 1.8 to convert it to the α,β,γ-tridentate complex. Any free ATP was removed by chromatography on Dowex 1 (Cl), and the tridentate complex was separated from polymer and residual bidentate complexes by chromatography on cycloheptaamylose (1.5 × 250 cm) equilibrated with 10 mM Pipes, pH 5.5, at 4 °C. The concentration of α,β,γ-tridentate CrATP was determined from its absorption spectrum (ϵ_{mM} = 22 at 430 nm and at 610 nm) (Cleland & Mildvan, 1979).

For NMR studies, adenylate kinase was exchanged into ^2H_2O by two cycles of lyophilization and redissolving. CrATP was concentrated and repeatedly diluted with ^2H_2O by rapid rotatory evaporation at 4 °C.

Proton NMR spectra were acquired on a Bruker WM-250 spectrometer operating in the Fourier transform mode at 250 MHz. Solvent water proton relaxation rate measurements were performed on the Bruker WM-250 and a Bruker WH-360 at 250 and 360 MHz, respectively.

Nuclear Overhauser effects were measured by using a low-power saturating radio-frequency pulse which was gated off immediately before the observe pulse. Difference spectra were generated by alternate acquisition of experimental and control spectra for which the saturating irradiation frequency was shifted on- and off-resonance, respectively. The other relevant parameters are given in the figure legends. Chemical shifts are reported in ppm downfield from external DSS.

Longitudinal relaxation rates were determined by the inversion–recovery method, and transverse relaxation rates were calculated from line-width measurements at half-height (Mildvan et al., 1980). Because of the limited amount of highly purified enzyme available, the correlation time for dipolar interaction between CrATP and the C2 proton of histidine-36 was determined from the frequency dependence of $1/T_{1p}$ of water protons in the same complex. This method has previously been shown to be appropriate for measuring correlation times of Cr(III) complexes (Mildvan et al., 1980; Cleland & Mildvan, 1979). Calculations of Cr(III)–proton distances were carried out according to the Solomon–Bloembergen–Morgan theory which has been thoroughly reviewed elsewhere (Mildvan & Gupta, 1978; Mildvan et al., 1980).

Results

Interaction of CrATP with Adenylate Kinase. The aromatic region of the proton NMR spectrum of adenylate kinase is shown in Figure 1. The single-proton resonances at 7.81 and 7.74 ppm have been assigned to the C2 protons of histidines-189 and -36, respectively (McDonald et al., 1975). Although the chemical shifts differ slightly from those reported by McDonald et al., they are easily distinguishable from one another by virtue of the decidedly different dependence of their chemical shifts on pH (McDonald et al., 1975). The chemical shift of the resonance of His-36 changes with pH while that of His-189 does not. Addition of α,β,γ-tridentate CrATP, a potent competitive inhibitor with respect to MgATP (Dunaway-Mariano & Cleland, 1980b), caused specific broadening of the resonance associated with His-36. The only other resonance that is detectably broadened by CrATP is near 6.55 ppm, which likely arises from the C4 proton of the same residue. This resonance was not well enough resolved for further study. Although other resonances may be broadened by the paramagnetic effect of the CrATP, none is sufficiently well resolved to make the effect visible. The magnitude of the increase in $1/T_2$ of the His-36 C2 resonance (Table I) sets a lower limit on the dissociation rate of CrATP of ≥400 s^{-1}.

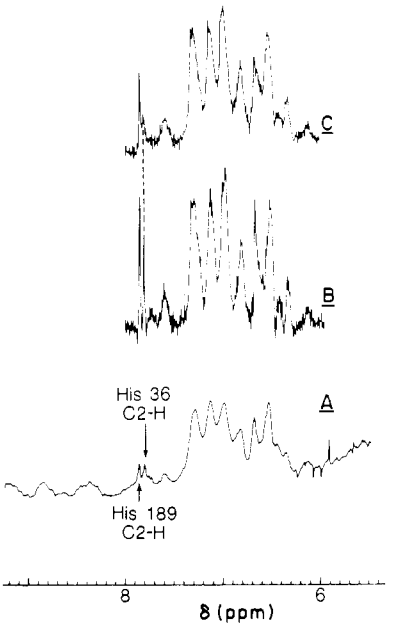

FIGURE 1: Aromatic region of the ^1H NMR spectrum of porcine adenylate kinase and broadening produced by α,β,γ-tridentate $Cr^{III}ATP$. (A) is the the ordinary spectrum and (B) the convolution difference NMR spectrum of a sample containing 0.72 mM adenylate kinase and 5 mM Tris-HCl, pH* 7.4. Spectrum C is the convolution difference spectrum after 22 μM CrATP was added. NMR parameters: 2.5-kHz spectral width, 16K time domain data points, 90° flip angle, and 5.0-s recycle time. The line broadenings for the convolution difference were 5.0 and 0.5 Hz, and the additive constant for the broadened spectrum was −0.85. The temperature for all experiments was controlled at 297 K.

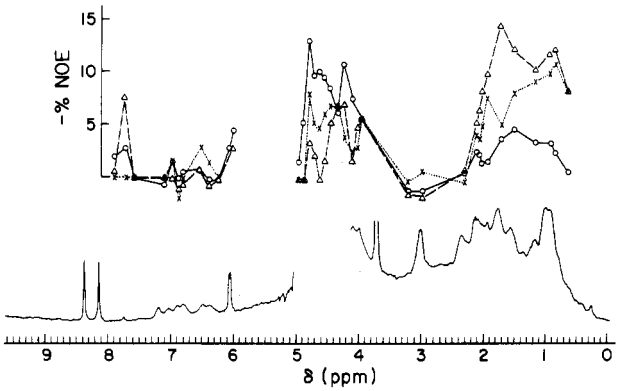

FIGURE 2: Action spectra of nuclear Overhauser effects to MgATP. The effect on adenine H8 is denoted by (○), on adenine H2, by (△); and on ribose H1′, by (×). A control spectrum is shown on the bottom. The sample contained 0.6 mM adenylate kinase, 5.0 mM ATP, 5.0 mM $MgCl_2$, and 5 mM Tris-HCl, pH* 7.25. NMR parameters: 2.5-kHz spectral width, 8K time domain points, 70° flip angle, 2.0-s recycle time, and 0.3-s preirradiation time.

In addition to increasing the $1/T_2$ of the His-36 C2 proton, CrATP also increases the $1/T_1$ (Table I). The increase in longitudinal relaxation rate is an order of magnitude lower than the minimum off rate calculated from $1/(fT_{2p})$ and is thus not exchange limited.

Paramagnetic effects on both T_1 and T_2 are reversed by MgATP. From the known dissociation constants of CrATP (Dunaway-Mariano & Cleland, 1980b) and MgATP (Hamada & Kuby, 1978), addition of 1.25 mM MgATP should have reduced the paramagnetic effects of CrATP on the relaxation rates by 32%. The observed reduction of $1/T_{1p}$ by 43% (Table I) agrees with prediction, confirming the binding of CrATP at the active site and further indicating that the binding of MgATP did not alter $1/T_1$ from its diamagnetic value. A similar reduction of $1/T_{2p}$ is seen, but the experimental error in this measurement is high (Table I).

The correlation time [$\tau_c = (5.5\text{–}8.0) \times 10^{-10}$ s; Table I] for the interaction between Cr(III) and the his-36 C2 proton determined in the same complex using the frequency dependence of $1/T_{1p}$ of solvent water protons is in the range of those found for other Cr(III) complexes (Cleland & Mildvan, 1979; Mildvan et al., 1980). By use of a correlation time of $(6.8 \pm 1.2) \times 10^{-10}$ s and $1/(fT_{1p}) = 24.6 \pm 5.0$ s^{-1}, the distance between the Cr(III) of bound CrATP and the His-36 C2 proton is found to be 12.9 ± 1.0 Å.

NOE Studies of the Adenylate Kinase–ATPMg Complex. Figure 2 shows the effect of irradiating various resonances in the spectrum of adenylate kinase in the presence of MgATP on the intensities of the H8, H2, and H1′ resonances of ATP. The effects appear to be selective; the "action spectra" of the three ATP resonances that could be monitored are different, and each action spectrum exhibits clear maxima. Thus, the phenomenon of spin diffusion (Kalk & Berendsen, 1976; Andree, 1978) does not play an important role in the effect probably due to the short duration (0.3 s) of the saturating pulse (Wagner & Wuthrich, 1979). This explanation is supported by other experiments, in which it was found that longer irradiation times (≥1 s) produced less distinct maxima and NOE's to many resonances of the protein.

Figure 3 shows the NOE's obtained by irradiation in the region of the His C2 resonances at a pH at which the resonance of His-36 is downfield from that of His-189, and the two signals are well separated. This experiment indicates that the NOE obtained to the adenine H2 proton originates from the His-36 C2 proton.

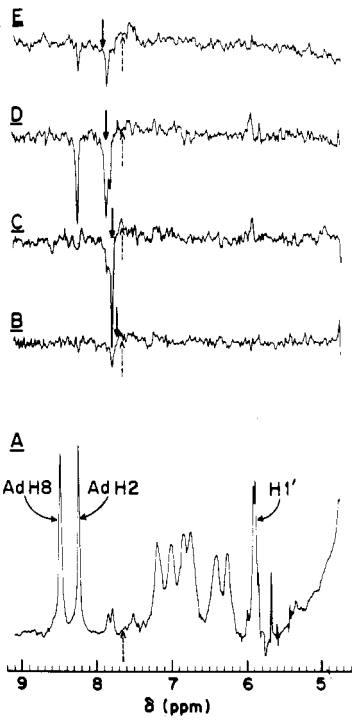

FIGURE 3: NOE between the His-36 C2 proton and adenine H2 of MgATP. The control spectrum (A) and the difference spectra between experimental and control ×4 are shown (B–E). The frequency of preirradiation shown by the solid arrows was varied, while the control preirradiation was held constant as shown by the dashed arrows. The sample contained 0.6 mM adenylate kinase, 5.0 mM ATP, 5 mM $MgCl_2$, and 5 mM Tris-HCl, pH* 7.0. At this lower pH*, the resonance of His-36 is downfield from that of His-189.

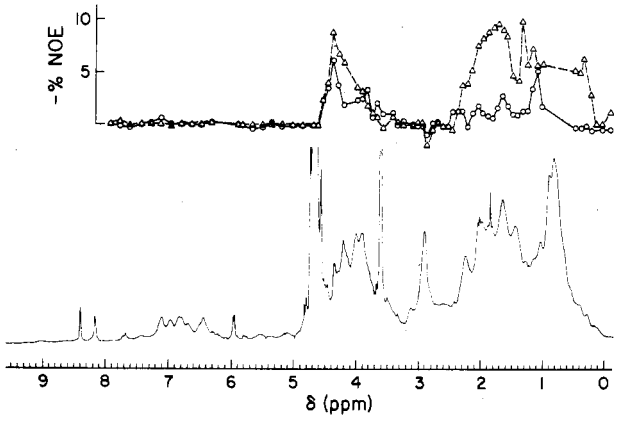

FIGURE 4: Action spectra of nuclear Overhauser effects to adenine H8 and adenine H2 of AMP. The symbols, conditions, and parameters are the same as those in Figure 2 except that 5.0 mM AMP was substituted for ATP and $MgCl_2$.

An experiment identical with that shown in Figure 2 but using MgGTP produced no detectable NOE's, possibly because of an unfavorable residence time in the active site (Bothner-By & Gassend, 1973).

NOE Studies of the Adenylate Kinase–AMP Complex. An experiment using AMP produced the action spectrum shown in Figure 4. The differences between the action spectra of MgATP adenylate kinase (Figure 2) and AMP adenylate kinase (Figure 4) demonstrate that the MgATP and AMP sites are indeed distinct and that the life time or extent of MgATP bound at the AMP site is insufficient to allow appreciable spin transfer from the spins of the protein.

Discussion

The chromium to proton distance obtained for the His-36

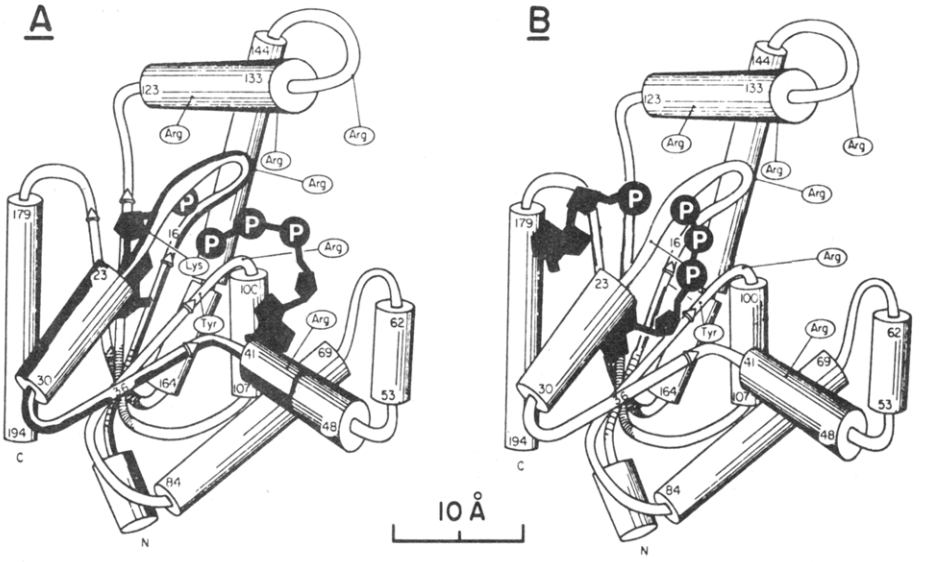

FIGURE 5: Alternative positions of MgATP and AMP in the active site of adenylate kinase. (A) Diagram showing the nucleotide binding sites in the crystallographic structure proposed by Pai et al. (1977). The positions of histidine-36 and of residues 1–44 have been highlighted. The ATP and AMP sites may have been reversed as discussed in the text. (B) Alternative positioning of ATP and AMP consistent with the present NMR data and with the binding studies of ATP and AMP analogues to peptides 1–44 and 171–193, respectively (Hamada et al., 1979).

C2 proton is qualitatively consistent with the line-broadening results of McDonald et al. (1975) induced by adding Mn^{2+} to the enzyme–ATPMg complex, although these authors could not make quantitative calculations because of the presence of MgATP in their sample. A primary Overhauser effect between two protons on a protein establishes a proximity of ≤ 5 Å between these protons (Rosevear et al., 1981). The NOE experiments, shown in Figures 2 and 3, therefore indicate that, in the adenylate kinase–ATPMg complex, the adenine H2 proton is within 5 Å of the His-36 H2 proton. Together with the large distance between the metal ion of CrATP and this proton, it is clear that the metal–ATP complex binds to the active site, with the adenine moiety near His-36 and the triphosphate chain extended away from this histidine residue, making the reasonable assumption that the competitive inhibitor, CrATP, and the substrate, MgATP, bind in the same way.

NOE's observed on the protons of the adenine groups from irradiation between 4 and 5 ppm in the adenylate kinase spectrum (Figures 2 and 4) could be either intermolecular from the spins of the protein or intramolecular from the ribose protons of bound nucleotide. The effect cannot arise from irradiation of free nucleotide because homonuclear NOE's in small molecules are positive. It is reasonable to assume that the effects come predominantly from the ribose protons of bound nucleotide (at least between 4.5 and 5 ppm) because of distance constraints and because the chemical shifts irradiated correspond roughly to those of the ribose protons. The ribose protons are generally ≤ 5 Å from the adenine H8 from model-building studies. These effects thus suggest an anti-conformation for the bound MgATP and serve to calibrate the NOE method for estimating other interproton distances on adenylate kinase. In favorable cases, the indidivual ribose–adenine proton distances can be measured by observing the rate at which NOE's build up compared to the rate at which the ribose protons become saturated (Rosevear et al., 1981). However, the individual ribose protons are not sufficiently well resolved in this case. The remainder of the effects in this region probably arise from the Cα protons of the protein.

Nuclear Overhauser effects on MgATP produced by irradiation of the protein proton resonances between 0 and 4 ppm (with maxima at 1.8 and 0.9 ppm) indicate that the H2 proton of MgATP is near at least one Arg Cβ (1.8 ppm) or Cγ (1.7 ppm), Lys Cδ (1.7 ppm), or Leu Cβ (1.6 ppm) and one or more Leu, Ile, or Val methyl groups (0.9 ppm). The NOE action spectra of the H1′ of ATP show similar maxima. Since the adenine ring must be near His-36, the residues most likely to contribute to the effect are Arg-44, Leu-37, and Ile-28.

The maxima in the NOE action spectra of MgATP at 1.8 and 0.9 ppm found in this study are indistinguishable from those found by James (1976) for creatine kinase, which were confirmed by Vasak et al. (1979). It is possible that these maxima at 1.8 and 0.9 ppm reflect at least the kinds of residues that are likely to be found in the MgATP binding sites of other kinases. A histidine has also been found near adenine H2 of ATP bound to pyruvate kinase (Meshitsuka et al., 1981).

A crystallographic study of the structure of nucleotide and inhibitor complexes of porcine adenylate kinase (Pai et al., 1977), summarized in the diagram of Figure 5A, has been interpreted to indicate that ATP binds with its triphosphate chain near His-36 and the adenine moiety in a hydrophobic pocket near Ile-92, Val-67 and -72, Leu-69, -73, and -26, and Arg-97. This assignment of the ATP binding site is based on the finding that the inhibitor salicylate was found to bind in this region. The inhibitor Ap_5A, without a metal ion, bound in a similar region, but its orientation was distorted since it bridged equivalent sites on separate enzyme molecules. Adenylate kinase and MgATP did not cocrystallize, and MgATP did not bind to adenylate kinase crystals of either form A or B. Although MnATP bound to pregrown crystals, the site at which it was bound was assigned to AMP. While the NOE's produced by irradiation in the aliphatic region of the spectra are not inconsistent with the results of the crystallographic study of salicylate binding, the NOE between His-36 and the H2 of MgATP require that they be ≤ 5 Å apart. This finding and the 12.9-Å distance from Cr(III) of CrATP to His-36 indicate that the metal–ATP complex does not bind in precisely the same way as salicylate. Moreover, the existence of a peptide fragment (residues 1–44 of the rabbit enzyme) (Hamada et al., 1979) and a synthetic nonapeptide (residues 32–40) that bind Mg^{2+} complexes of 1,N^6-etheno analogues of ATP and ADP with the same stoichiometry and affinity as the native enzyme argue against the involvement

of the residues implicated in the crystallographic study. It is therefore possible that the assignments of the ATP site and the AMP site have been reversed in the crystallographic study illustrated in Figure 5A. While reversing the ATP and AMP sites in Figure 5A would satisfy the NMR data, it would not satisfy the observation (Hamada et al., 1979) that a peptide of residues 171–193 binds AMP. An alternative placement of ATP and AMP which would provide a better fit to the existing data is suggested in Figure 5B. The binding site for ATP partially overlaps with that proposed in the crystallographic study and is therefore consistent with the action of salicylate as a competitive inhibitor.

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Isolation and Characterization of Diadenosine 5′,5‴-P^1,P^4-Tetraphosphate Pyrophosphohydrolase from *Physarum polycephalum*†

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ABSTRACT: A new enzyme that hydrolyzes diadenosine 5′,5‴-P^1,P^4-tetraphosphate has been purified by a factor of 250 from the acellular slime mold *Physarum polycephalum*. Activity was assayed radioisotopically with [^{3}H]Ap_4A. Isolation of the enzyme was facilitated by dye–ligand chromatography. The enzyme symmetrically hydrolyzes Ap_4A to ADP and exhibits biphasic kinetics for the substrate with values for the apparent K_m of 2.6 μM and 37 μM. The two values of V_{max} differ by a factor of 10. Mg^{2+}, Ca^{2+}, and other divalent cations inhibit the activity with 40–80% inhibition occurring at 0.5 mM. Mg^{2+}, at 0.5 mM, decreases both values of V_{max} by 50%, decreases the low K_m value by about 30%, and increases the high K_m value by about 100%. (Ethylenedinitrilo)tetraacetic acid (EDTA) and [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), at 10 mM, inhibit the activity by 50%. ADP, ATP, Ap_4, and Gp_4 are equipotent inhibitors with 50% inhibition occurring at 30 μM. AMP is a relatively weak inhibitor. The molecular weight of the enzyme is 26 000 on the basis of elution of activity from a calibrated Sephadex G-75 column.

Diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A)[1] is synthesized from ATP and bound aminoacyl-AMP by some tRNA synthetases from *Escherichia coli*, yeast, and *Physarum polycephalum* (Zamecnik et al., 1966; Randerath et al., 1966; Plateau et al., 1981; Goerlich et al., 1982). Broad species distribution of this reaction and its occurrence in vivo have not been demonstrated, but Ap_4A has been detected in both procaryotic and eucaryotic species at concentrations of 10^{-8}–10^{-7} M (Zamecnik, 1969; Rapaport & Zamecnik, 1976). Zamecnik and co-workers proposed that Ap_4A may be a regulator by which the status of protein synthesis influences

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[1] Abbreviations: Ap_nA, diadenosine 5′,5‴-P^1,P^n-polyphosphate, n = 2–6; Ap_4, adenosine tetraphosphate; Gp_4, guanosine tetraphosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; DEAE, diethylaminoethyl; PEI, poly(ethylenimine); NADH, reduced nicotinamide adenine dinucleotide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid.

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