Complexes of Yeast Adenylate Kinase and Nucleotides Investigated by ^{1}H NMR

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ABSTRACT: The role of one of the histidine residues present in many adenylate kinases (H36 in the porcine cytosolic enzyme) is highly disputed. We thus studied the yeast enzyme (AK_{ye}) containing this His residue. AK_{ye} is highly homologous to the *Escherichia coli* enzyme (AK_{ec}), a protein that is already well characterized by NMR [Vetter et al. (1990) *Biochemistry 29*, 7459–7467] and does not contain the His residue in question. In addition, discrepancies between solution structural and X-ray crystallographic studies on the location of the nucleotide binding sites of adenylate kinases are clarified. One- and two-dimensional nuclear magnetic resonance (NMR) spectroscopy was used to investigate AK_{ye} and its complex with the bisubstrate analogue P^1,P^5-bis(5′-adenosyl)pentaphosphate (AP_5A). The well-resolved spectra of AK_{ye} allowed identification of nearly all detectable resonances originating from aromatic side chain protons (12 out of 15 spin systems). From these studies, all aromatic residues of AK_{ec} involved in the binding of $ATP{\cdot}Mg^{2+}$ have functional analogues in AK_{ye}. The AMP site seems to make no contacts to aromatic side chains, neither in the $AK_{ye}{\cdot}AP_5A{\cdot}Mg^{2+}$ nor in the $AK_{ec}{\cdot}AP_5A{\cdot}Mg^{2+}$ complexes, so that it is presently not possible to localize this binding site by NMR. The ATP site of AK_{ye} is located near residues W^{210} and H^{143} in a position similar to the ATP site of the *E. coli* enzyme. In combination with the recent X-ray results on the AP_5A complexes AK_{ye} and AK_{ec} and the GMP complex of guanylate kinase [Stehle, T., & Schulz, G. E. (1990) *J. Mol. Biol. 221*, 255–269], the latter one leading to the definition of the monophosphate site, the problem of the location of the nucleotide sites can be considered to be solved in a way contradicting earlier work [for a review, see Mildvan, A. S. (1989) *FASEB J. 3*, 1705–1714] and denying the His residue homologous to H36 in porcine adenylate kinase a direct role in substrate binding.

Adenylate kinases (AK,[1] ATP:AMP-transphosphorylase, EC 2.7.4.3) are ubiquitous phosphokinases with relatively low molecular weights (21 000–28 000) catalyzing the reaction (Noda, 1973)

$$AMP + Mg^{2+}{\cdot}ATP \leftrightarrow ADP + Mg^{2+}{\cdot}ADP$$

The sequences of about 10 adenylate kinases have been determined. The X-ray structure of porcine adenylate kinase is known (Schulz et al., 1973; Heil et al., 1974; Dreusicke et al., 1988). So far it was not possible to cocrystallize any of the adenylate kinases with their substrates. Thus, the structure of the yeast adenylate kinase (Egner et al., 1987) and the *Escherichia coli* adenylate kinase complex (Müller & Schulz, 1988) with the supposed bisubstrate analogue P^1,P^5-bis(5′-adenosyl)pentaphosphate (AP_5A) (Lienhard & Secemski, 1973) has to serve as a model for the substrate-loaded enzyme; in fact, it was shown earlier by NMR that the porcine $AK{\cdot}AP_5A{\cdot}Mg^{2+}$ complex and the $AK_{ec}{\cdot}AP_5A{\cdot}Mg^{2+}$ complex can serve as decent analogues for the enzyme with both substrates bound (Rösch et al., 1989; Vetter et al., 1990). The position of AP_5A in the two known X-ray structures of $AK{\cdot}AP_5A$ complexes is identical.

In contrast, the X-ray crystallographic structure of guanylate kinase with the monophosphate substrate GMP bound could be determined very recently (Stehle & Schulz, 1990). One of the adenosine binding sites of the adenylate kinases as derived from the X-ray crystallographic studies of the $AK{\cdot}AP_5A$ complex (Egner et al., 1987; Müller & Schulz, 1988) indeed corresponds structurally to this GMP site (Stehle & Schulz, 1990).

E. coli, carp, rabbit, porcine, and chicken adenylate kinases have also been extensively investigated by NMR, one of the major aims being the localization of the two substrate sites in solution [Mildvan (1989) and references therein; Tian et al., 1988; Rösch et al., 1989; Vetter et al., 1990). Recent NMR experiments with complexes of AK_{ec} and nucleotides as well as AP_5A allowed identification of the ATP site in solution as being identical with the location of one of the adenosine moieties of AP_5A in the crystal (Vetter et al., 1990; Müller & Schulz, 1988). This site does not correspond to the GMP site of the guanylate kinase structure (Stehle & Schulz, 1990), so that the combination of the X-ray and NMR experiments leads to an unequivocal determination of both nucleotide sites in AK_{ec}.

Here we present new evidence on the location of the nucleotide binding sites of adenylate kinases. In order to clarify the role of a His residue considered crucial by many investigators [H36 in the porcine enzyme sequence numbering;

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[1] Abbreviations: ADP, adenosine diphosphate; AK, adenylate kinase; AK_1, cytosolic adenylate kinase; AK_{ec}, *E. coli* adenylate kinase; AK_{ye}, yeast adenylate kinase; AMP, adenosine monophosphate; AP_5A, P^1,P^5-bis(5′-adenosyl)pentaphosphate; ATP, adenosine triphosphate; COSY, correlated spectroscopy; 1D and 2D, one-dimensional and two-dimensional; DQF-COSY, double quantum filtered COSY; DSS, 2,2-dimethyl-2-silapentanesulfonate; DTE, dithioerythritol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-pressure liquid chromatography; K_d, dissociation constant; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total coherence spectroscopy; Tris, tris(hydroxymethyl)aminomethane.

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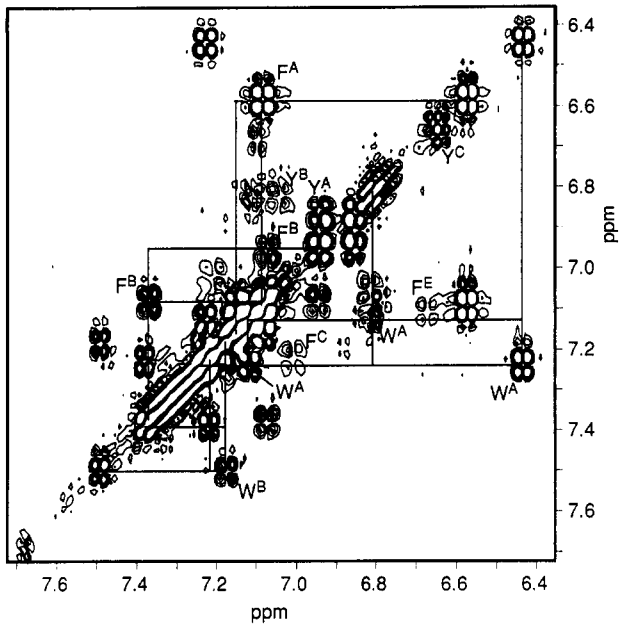

FIGURE 1: Aromatic ring proton resonance region of the DQF-COSY spectrum of yeast adenylate kinase. [AK_{ye}] = 1.6 mM; HEPES buffer, 50 mM, pH 8.0.

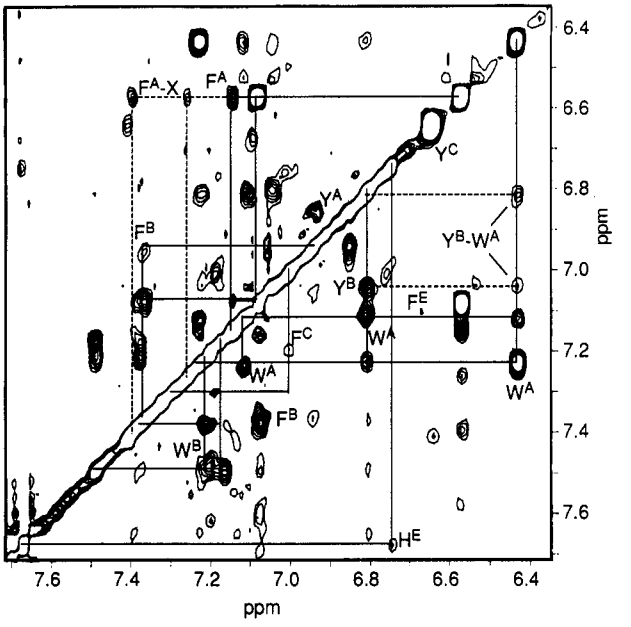

FIGURE 2: Aromatic ring protein resonance region of the NOESY spectrum of AK_{ye}. [AK_{ye}] = 1.6 mM; HEPES buffer, 50 mM, pH 8.0.

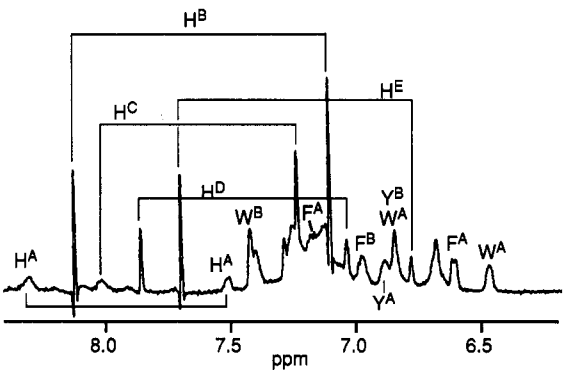

FIGURE 3: Aromatic ring proton resonance region of the 1D spectrum of AK_{ye}. [AK_{ye}] = 1.6 mM; HEPES buffer, 50 mM, pH 8.0.

reviewed in Mildvan (1989)], we used the yeast enzyme in our studies. This protein is highly homologous to the *E. coli* enzyme, but does contain a histidine residue corresponding to H36 in the porcine enzyme, which the *E. coli* enzyme is lacking. We also present some additional data on the porcine AK·nucleotide complexes, forcing us to revise our earlier assignment of the H36 C2-H resonance in the AK_1·AP_5A·Mg^{2+} complex (Rösch et al., 1989), as already indicated in a recent paper (Vetter et al., 1990). Our misassignments seemed to corroborate earlier observations based on magnetic resonance and peptide binding studies, a claim that is no longer tenable in light of the present results.

Materials and Methods

Preparation Procedures. Adenylate kinases were prepared and activity was measured essentially as described earlier (Barzu & Michelson, 1983; Ito et al., 1980; Berghäuser & Schirmer, 1978). The proteins were stored as lyophilized powders at 253 K after the preparation. The nucleotide concentration of the preparations was checked by HPLC before use and found to be below the limits of detection.

Nucleotides were obtained from Sigma at the highest purity commercially available. AP_5A was prepared according the Feldhaus et al. (1975) with minor modifications. HPLC analysis showed the purity of the AP_5A solutions to be better than 98%.

For the NMR experiments, residual H_2O was exchanged with D_2O by addition of D_2O (99.8%, from Sigma) to a preweighed amount of protein, followed by overnight storage at room temperature in order to exchange labile amide protons. This was followed by lyophilization and subsequent solution in 99.96% D_2O ("100% D_2O" from Sigma) to the desired concentration. The accurate protein concentration was determined before the NMR experiments according th Ehresman et al. (1973). Final buffer concentration was about 50 mM in all experiments. In titration experiments, nucleotides and AP_5A were added to the enzyme solution from 100 and 40 mM stock solutions, respectively. All stock solutions were stored at 253 K.

NMR Experiments and Data Evaluation. NMR experiments were performed on commercial Bruker AM 500 and AM 600 spectrometers operating at proton resonance frequencies of 500 and 600 MHz, respectively, employing standard procedures throughout [for details, see Vetter et al. (1990)].

Results

NOESY and COSY Experiments with AK_{ye}. The spectral region of the aromatic side-chain proton resonances of the DQF-COSY spectrum of AK_{ye} allows detection of resonances for nearly the complete set of aromatic spin systems of the enzyme (Figure 1). The typical resonance patterns were found for the two tryptophan, the three tyrosine, and the five phenylalanine ring spin systems, although three of the phenylalanine side chains give rise to resonances of only very low intensity, one set of cross peaks (F^D) being at the very limit of detection in the contour plots. One of the tyrosine cross peaks is located very close to the diagonal (Y^C). Two interresidual NOESY cross peaks connect the spin systems of Y^B and W^A, and two others connect one of the ring proton resonances of F^A with two diagonal peaks (labeled F^A-X in Figure 2), most probably representing F^D.

The resonances downfield from 7.5 ppm in the spectrum are easily identified as originating from histidine imidazole ring protons by their chemical shifts and, in the cases of H^B, H^D, and H^E, by their small resonance line widths (Figure 3). The connectivities of the C2-H and the C4-H resonances of all

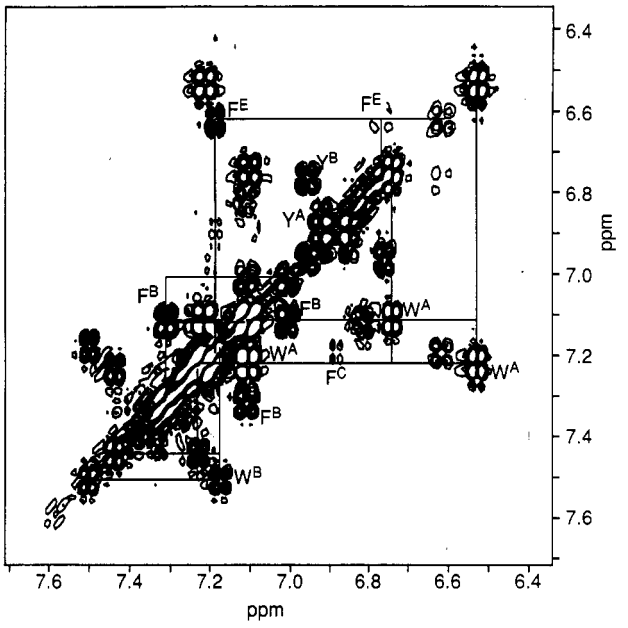

FIGURE 4: Aromatic ring proton resonance region of the DQF-COSY spectrum of the AK_{ye}·AP_5A·Mg^{2+} complex. [AK_{ye}] = 1.6 mM; [AP_5A] = 3 mM; [$MgCl_2$] = 10 mM; HEPES buffer, 50 mM, pH 8.0.

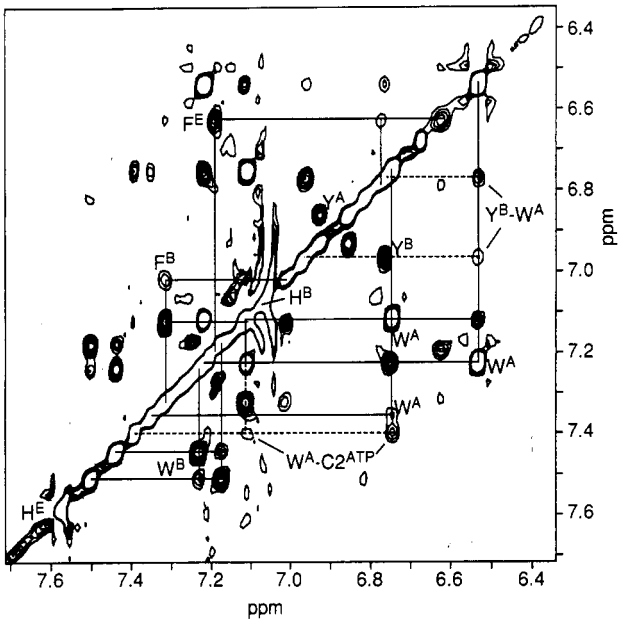

FIGURE 5: Aromatic ring proton resonance region of the NOESY spectrum of the AK_{ye}·AP_5A·Mg^{2+} complex. [AK_{ye}] = 1.6 mM; [AP_5A] = 3 mM; [$MgCl_2$] = 10 mM; HEPES buffer, 50 mM, pH 8.0.

imidazole rings as derived from the NOESY spectra and some connectivities of resolved aromatic spin systems are shown in Figure 3.

NOESY and COSY Experiments with the AK_{ye}·AP_5A·Mg^{2+} Complex. The DQF-COSY spectrum of the AK_{ye}·AP_5A·Mg^{2+} complex (Figure 4) shows minor changes when compared to the spectrum of inhibitor-free AK_{ye}. Most of the resonances are easily identified by using the additional information of the NOESY spectrum (Figures 5 and 6). The numbers of detectable interresidual NOESY cross peaks is increased from four to six compared to the spectrum of uncomplexed AK_{ye} (Figure 2). Three new cross peaks indicate dipolar interaction between side-chain protons of H^C and W^A (Figure 6), two signals show the proximity of Y^B and W^A, and one NOE cross

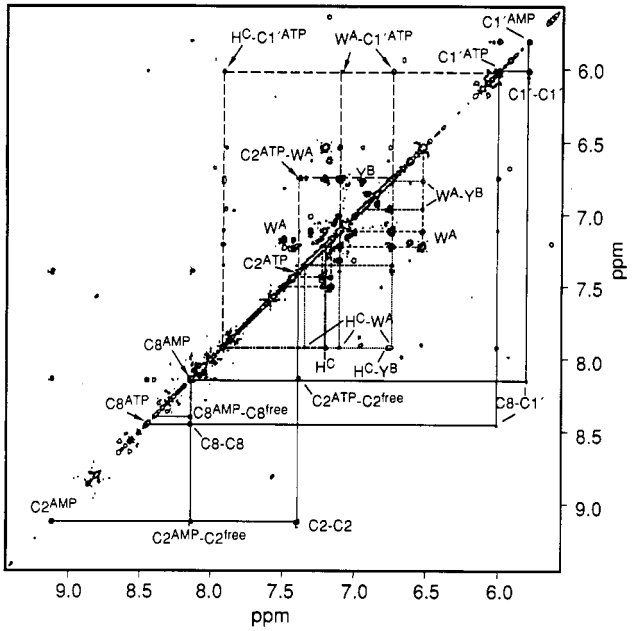

FIGURE 6: Extended part of the aromatic ring proton resonance region of the NOESY spectrum of the AK_{ye}·AP_5A·Mg^{2+} complex. [AK_{ye}] = 1.6 mM; [AP_5A] = 3 mM; [$MgCl_2$] = 10 mM; HEPES buffer, 50 mM, pH 8.0.

peak connects resonances of the imidazole ring protons of H^C with the resonances of the ring protons of Y^B (Figure 6). This leads to the conclusion that H^C, W^A, and Y^B are arranged in a cluster with interresidual distances of less than 0.4 nm (Wüthrich, 1986).

The intense resonances from the side-chain protons of F^A in the spectrum of the inhibitor-free enzyme are either not identifiable or missing in the 2D spectra of the AK_{ye}·AP_5A·Mg^{2+} complex, and thus, the interresidual cross peaks to the resonance assumed to originate from F^D also disappeared. The distorted peak shape of H^B (Figure 5) is due to a change of the chemical shift of this resonance during the 2D experiment, probably an effect of minor changes of the pH value in the course of the experiment. However, chemical shifts of other resonances did not change during the experiment within the limits of error.

The ribose C1′-H and the adenosine C2-H/C8-H resonance region of the NOESY spectrum of the AK_{ye}·AP_5A·Mg^{2+} complex (Figure 6) shows a characteristic pattern of AP_5A proton resonances very similar to the pattern observed with the AK_{ec}·AP_5A·Mg^{2+} complex (Vetter et al., 1990). A set of signals for each adenosine moiety of the enzyme-bound AP_5A consisting of the resonances of the ribose C1′ proton and the adenine C2 and C8 protons can be identified. The sites represent the ATP site and the AMP site, respectively, according to the results by Vetter et al. (1990). The corresponding protons of the two sites are connected via strong cross peaks caused by the reversal of the two adenosine moieties of AP_5A in the bound state in a process slow on the NMR time scale. These cross peaks are labeled C2–C2, C8–C8, and C1′–C1′ in Figure 6. The intense cross peak in the NOESY spectrum between the C2-H resonances of the two adenosine moieties of bound AP_5A and the corresponding C2-H resonances of free Ap_5A, which are degenerate, ($C2^{AMP}$–$C2^{free}$ and $C2^{ATP}$–$C2^{free}$ in Figure 6) is observed only at ligand concentrations in excess of the protein concentration. The same is observed for the C8-H resonance representing the AMP site as shown below (Figure 6). The reason for our failure to detect the cross peak between the $C8^{ATP}$-H resonance and the C8-H resonance of free AP_5A may be the close spacing of the chemical shifts,

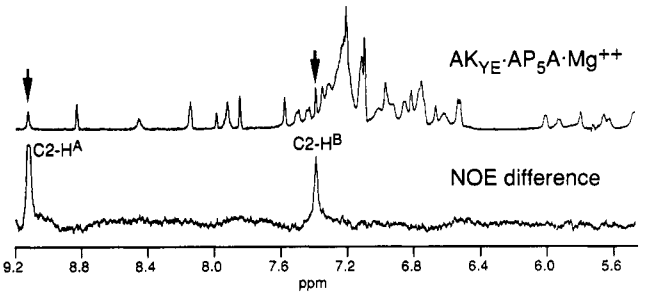

FIGURE 7: Upper trace: aromatic ring proton resonance region of the AK_{ye}·AP_5A·Mg^{2+} complex. Lower trace: difference spectrum between a spectrum irradiated at the frequency of the low-field base C2-H. [AK_{ye}] = 1.5 mM; [AP_5A] = 1.5 mM; [$MgCl_2$] = 5 mM; HEPES buffer, 50 mM, pH 8.0.

Table I: Chemical Shift Values of Adenylate Kinase Bound Nucleotides

		porcine AK_1	AK_{ec}	AK_{ye}	free
AMP	C1′H	6.03	5.98	nd[a]	6.13
	C2H	8.65	8.82	nd	8.22
	C8H	8.58	8.47	nd	8.59
ATP	C1′H	6.09[b]	6.11	nd	6.20
	C2H	ca. 8.4 [b]	7.80	nd	8.26
	C8H	ca. 8.5[b]	8.42	nd	8.55
Ap_5A·Mg^{2+} (AMP)	C1′H	5.8	5.87	5.84	
	C2H	8.9	9.14	9.14	
	C8H	8.4	8.26	8.17	
AP_5A·Mg^{2+} (ATP)	C1′H	6.06	6.08	6.04	
	C2H	8.26	7.42	7.42	
	C8H	8.37	8.39	8.48	
AP_5A	C1′H				6.06
	C2H				8.12
	C8H				8.44

[a] nd, not determined. [b] Determined in the presence of Mg^{2+}.

leading to a location of the expected cross peak too close to the diagonal. The identification of the two C2-H resonances from bound AP_5A can be corroborated by a 1D saturation transfer experiment: Irradiation of the $C2^{AMP}$ proton resonance at 9.1 ppm causes a large intensity change of the exchange-coupled $C2^{ATP}$ proton resonance at 7.4 ppm (Figure 7). C8 and C1′ protons can be identified as belonging to the same site with the help of connecting NOESY cross peaks (labeled C8–C1′ in Figure 6).

The intermolecular NOESY cross peaks between AP_5A and aromatic side-chain protons of the protein connect the high-field C1′-H and W^A, the same C1′-H and H^C, and $C2^{ATP}$-H and W^A (Figure 6). The assignment of the two adenosine C1′-H and C8-H resonances in the bound state to the two different sites is now obvious: W^A side-chain proton resonances show NOESY cross peaks to the $C2^{ATP}$-H resonance as well as to the high-field C1′-H resonance, rendering the assignment of this resonance to $C1'^{ATP}$-H. This, in turn, allows assignment of the C8-H resonances (Figure 6). In addition, these cross peaks indicate that the ATP moiety of AP_5A is located close to the side chains of W^A, H^C, and Y^B. The chemical shift values of the C1′-, C2-H, and C8-H resonances of both adenosine moieties of bound AP_5A are listed in Table I.

Similar to the results obtained with AK_{ec}, the resonances of $C1'^{AMP}$-H, $C2^{AMP}$-H, and $C8^{AMP}$-H of AP_5A show no NOESY cross peaks in the aromatic proton resonance region.

Titration of AK_{ye} with AP_5A. Addition of AP_5A and excess $MgCl_2$ to a solution of AK_{ye} causes some rearrangements of the resonances of the aromatic side chains (Figure 8). All resonances are in slow exchange during the titration, so that it is not possible to follow the course of most signals. The resolution of the spectrum is improved after addition of AP_5A.

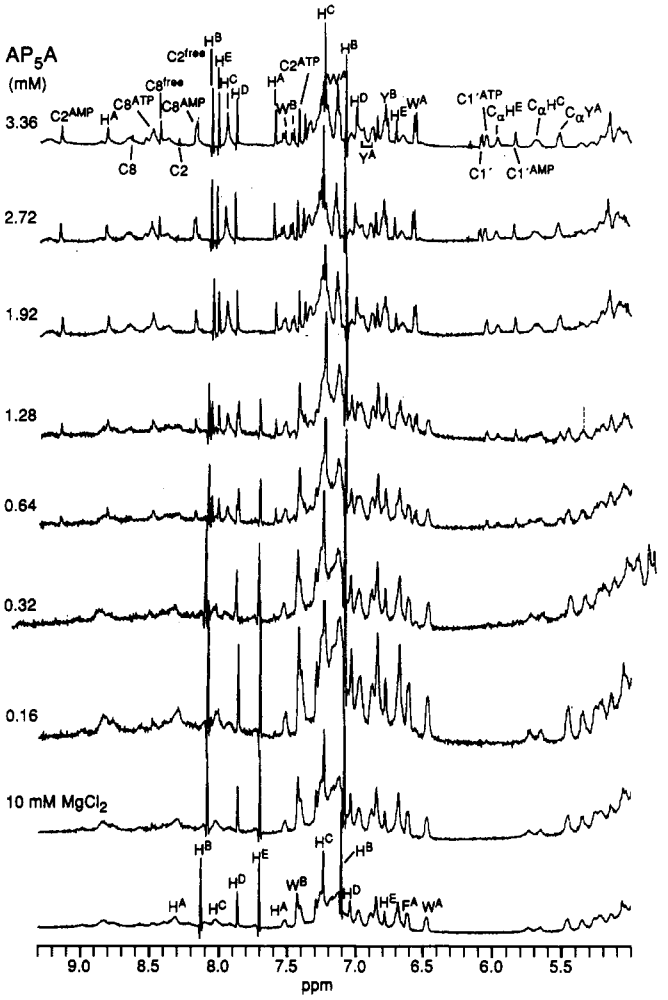

FIGURE 8: Aromatic ring proton resonance region of the spectrum of AKye and the AK_{ye}·AP_5A·Mg^{2+} complex at concentrations of AP_5A as indicated. [AK_{ye}] = 1.6 mM; [$MgCl_2$] = 10 mM; HEPES buffer, 50 mM, pH 8.0.

At AP_5A concentrations of about 2.7 mM, three new resonances at 8.6, 8.2, and 6.1 ppm (labeled C8, C2, and C1′ in Figure 8, upper trace) appear. These chemical shift values are identical with those of free adenine mononucleotides such as ATP or AMP. In addition, the presence of AMP, ADP, and ATP in a concentration amounting to about 30% of the total nucleotide concentration in the sample was evident from HPLC measurements. The sudden appearance of these nucleotides and their absence in the enzyme free stock solution as checked by HPLC indicate their origin as degradation products of AP_5A in the sample.

These nucleotides are not expected to participate in binding to the enzyme, because the dissociation constants expected on the basis of the corresponding values for AK_{ec} and other adenylate kinases are much higher than the dissociation constant of AP_5A (K_d values for AK_{ec}: AMP, 520 μM; ADP, 4.0 μM; ATP·Mg^{2+}, 85 μM; AP_5A·Mg^{2+}, 0.015 μM; Reinstein et al., 1990). Since this phenomenon does not appear at substoichiometric concentrations of AP_5A, AK_{ye} seems to protect the analogue from being degraded. Thus we conclude that contaminating proteins cause the generation of mononucleotides from AP_5A. The same observation was made in experiments with porcine AK.

Reassignment of the H36 C2-H Resonance in AK_1. The location of the ATP site as determined here (see Discussion) and in Vetter et al. (1990) contradicts earlier NMR and peptide-binding studies (Mildvan, 1989; Smith & Mildvan,

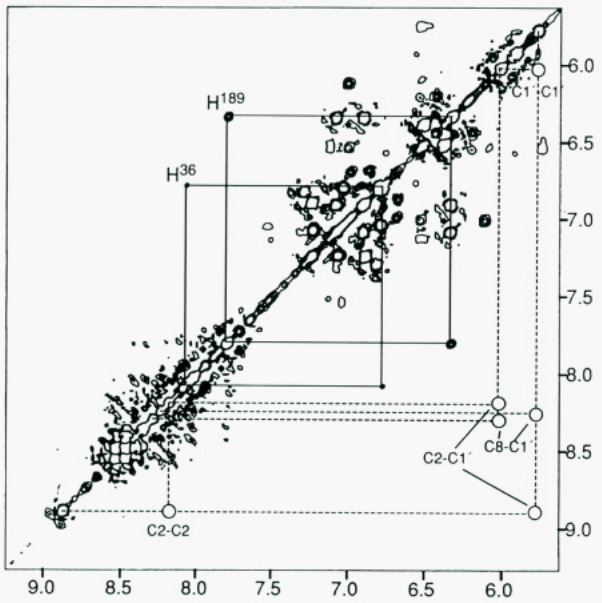

FIGURE 9: 600-MHz TOCSY spectrum of the porcine AK_1·AP_5A·Mg^{2+} complex (aromatic region). [AK_1] = 1.5 mM; [AP_5A] = 3 mM; [$MgCl_2$] = 2.5 mM; HEPES buffer, 50 mM, pH 8.0. The AP_5A NOE cross-peak pattern derived from the corresponding NOESY spectrum is indicated by circles in the lower right.

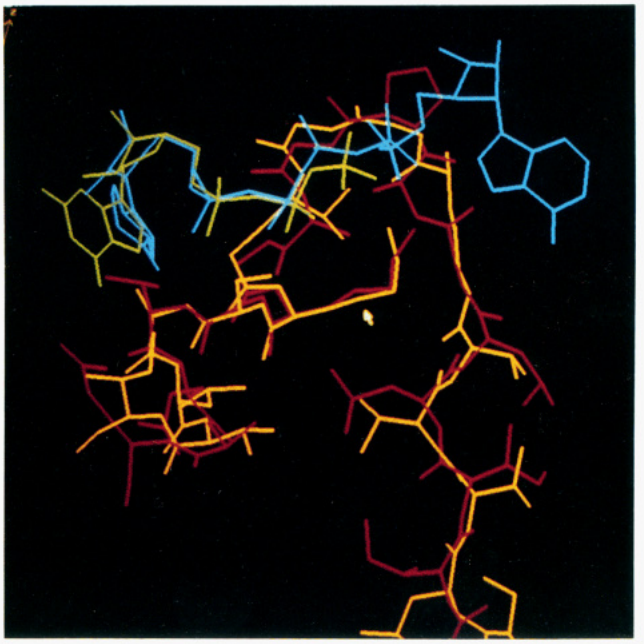

FIGURE 10: Comparison of the G-loop region of the *E. coli* AK·AP_5A·Mg^{2+} complex (red, protein; blue, AP_5A; courtesy of G. Schulz, Freiburg, FRG) and the corresponding region of the oncogene product p21·GTP·Mg^{2+} complex [yellow, protein; green, GTP; courtesy of Pai et al. (1989)].

1982; Hamada et al., 1979; Fry et al., 1985, 1987, 1988). Thus, our assignment of the crucial C2-H resonance of H36 in the porcine AK_1·AP_5A·Mg^{2+} complex based on these earlier results, which we presented recently (Rösch et al., 1989), needed closer inspection. Consequently, we performed a TOCSY experiment under optimized conditions at 600 MHz of the porcine AK_1·AP_5A·Mg^{2+}. In this experiment we could locate the C2-H resonance of H36 in the spectrum unequivocally (Figure 9), which leads to a revision of the assignments in Rösch et al. (1989). The cross peak formerly suggested to represent an NOE between the imidazole C2-H resonance of H^{36} and the low-field adenosine C2-H resonance of bound AP_5A is thus shown to represent an exchange cross peak between the two adenosine C2-H resonances of bound AP_5A. This view is also supported by NMR experiments with the chicken adenylate kinase mutant H36Q [Yuan and Tsai, unpublished results, as quoted in Sanders et al. (1989)] and further experiments with porcine and carp adenylate kinases (Vetter et al., unpublished results).

Discussion

Comparison of our NMR results with the published X-ray data of AK_{ec} and AK_{ye} containing AP_5A (Egner et al., 1987; Müller & Schulz, 1988) and with the sequence alignment according to Haase et al. (1989) allows suggestions for the sequence-specific assignment of some important residues of AK_{ye}. We had to draw our conclusions from a tertiary structure alignment of AK_{ec} and AK_{ye}, as the coordinates for the X-ray structure of yeast adenylate kinase (Egner et al., 1987) were not available. From this alignment it is obvious that the resonances of the protons of the W^{210} side chain are the only ones of the tryptophan type that can give rise to NOESY cross peaks to resonances from the protons of side chains of a histidine–tyrosine pair as it is observed in our NOESY experiments. The histidine–tyrosine pair is thus easily identified as Y^{142} and H^{143}. It follows that the ATP·Mg^{2+} site of AK_{ye} is located close to the side chains of residues W^{210}, H^{143}, and Y^{142}. This is in agreement with the position of one of the two adenosine sites of AP_5A in the X-ray structure of the AK_{ye}·AP_5A·Mg^{2+} complex (Egner et al., 1987). H^{143} and Y^{142} are homologous to AK_{ec} H^{134} and Y^{133}, which were suggested to form part of the ATP·Mg^{2+} binding structure of AK_{ec} (Vetter et al., 1990). W^{210} may then play the functional role of AK_{ec} F^{19}, although these residues are not homologous in the sequence alignment.

Thus we have complete agreement not only between the NMR results on the AK_{ec}·AP_5A·Mg^{2+} and the AK_{ye}·AP_5A·Mg^{2+} complexes but also between the NMR results and the X-ray crystallographic results. It is also clear from this and other work, in particular work relying on single point mutants [reviewed in Yan and Tsai (1991) and Kim et al. (1990)], that both the location of the C2-H of the ATP adenosine moiety close to the imidazole C2-H of H36 in AK_1 and the location of the AMP site according to work reviewed by Mildvan (1989) is no longer tenable.

The relative position of the triphosphate and the so-called G-loop (Dreusicke & Schulz, 1986, and references therein; Möller & Amons, 1985) is virtually identical for adenylate kinase and the H-*ras* oncogene product p21 (Figure 10). This loop comprises amino acids 7–14 in AK_{ec}, amino acids 11–18 in AK_{ye}, and amino acids 10–17 in p21. From a structural as well as a functional point of view, the two proteins do not have much in common, so that this observation is strongly suggesting the correctness of the assumption that the G-loop structure is a common motif of triphosphate binding proteins in general [for a review of these aspects, see Saraste et al. (1990)].

Acknowledgments

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Forschung, Braunschweig, FRG. The coordinates of the AK_{gc}·AP_5A·Mg^{2+} complex were kindly provided by Prof. Dr. G. Schulz, Freiburg, FRG, prior to their submission to the protein data bank. Excellent technical assistance from Ms. Birgit Brandmeier is gratefully acknowledged.

Registry No. AK, 9013-02-9; His, 71-00-1; ATP, 56-65-5; AMP, 61-19-8.

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