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# Proton-Nuclear Magnetic Resonance Studies of the Aromatic Spin Systems of *Escherichia coli* Adenylate Kinase

*Escherichia coli* adenylate kinase has a very well resolved proton nuclear magnetic resonance spectrum in the region containing signals from aromatic amino acid side-chains. We found that the protein is structurally stable over a wide pH range and renatures spontaneously after acidic as well as basic denaturation. Only one out of the three histidyl imidazole rings titrates on changing the pH and has a p*ka* value of 7·6. Two-dimensional nuclear magnetic resonance spectroscopy studies allowed use to identify most of the enzyme's aromatic spin systems, and by investigation of a mutant protein we were able to assign the aromatic part of the spin system of $Tyr^{24}$ unambiguously.

Adenylate kinases (AdK†, ATP:AMP-phosphoryltransferase, EC 2.7.4.3) are the smallest phosphorylkinases known to date with molecular weights as low as 21,700 (porcine AdK; *Escherichia coli* AdK: 23,500). In the presence of divalent metal cations they catalyse the transfer of a phosphoryl group from a nucleoside-triphosphate to a nucleoside-monophosphate:

$$Mg^{2+} \cdot ATP + AMP <\text{----}> Mg^{2+} \cdot ADP + ADP.$$

Among the best characterized adenylate kinases are the porcine, yeast, and *E. coli* enzymes. The primary structures of all three proteins are known (Heil *et al.*, 1974; Tomasselli *et al.*, 1986; Brune *et al.*, 1985), as well as the X-ray structures of the porcine and the yeast enzymes (Schulz *et al.*, 1974; Egner *et al.*, 1987). Unfortunately, X-ray studies could not unequivocally determine the location of the nucleotide binding sites on either the porcine or the yeast enzyme, as the porcine enzyme does not co-crystallize with its substrates or their analogues (Pai *et al.*, 1977) and although the yeast adenylate kinase can be co-crystallized with $Ap_5A$ (a strong inhibitor of the porcine adenylate kinase reaction (Lienhard & Secemski, 1973)), the $Ap_5A$ binding sites in the crystal do not necessarily reflect both substrate binding sites (Egner *et al.*, 1987).

In the case of porcine adenylate kinase, early proton n.m.r. studies led to sequence-specific assignment of the C-2 imidazole protons of the protein's two His residues (McDonald *et al.*, 1975). However, conclusions from recent n.m.r. experiments that were aimed at determining the location of the nucleotide binding sites are ambiguous (Smith & Mildvan, 1982; Fry *et al.*, 1985) as no additional spin system identifications, let alone sequence-specific assignments, have been made for the protein.

In order to remedy this situation we have applied two-dimensional $^1H$-n.m.r. techniques (Ernst *et al.*, 1987; Wüthrich, 1986) and genetic engineering methods to the study of *E. coli* AdK. We demonstrate that by this approach not only spin system identifications, but also sequence-specific assignments of aromatic residues other than histidines are possible in proteins as large as 23,000 $M_r$.

The *E. coli* adenylate kinase was purified from an overproducing strain using standard methods (Brune *et al.*, 1985; Barzu & Michelson, 1983) and the enzyme activity was checked with the assay described by Berghäuser & Schirmer (1978). $H_2O$ was exchanged for $D_2O$ by lyophilization of the protein solutions and subsequent redilution with $D_2O$. The lyophilization process was then repeated, and the protein dissolved in $D_2O$ to the desired concentration. The pH of enzyme solutions was adjusted with NaOD and DCl, and reported values are direct meter readings. The final phosphate buffer concentration in the samples was approximately 50 mM. The nucleotide content of all samples was checked by high pressure liquid chromatography before use; the nucleotide concentration was found to be less than 2% of the protein concentration in all cases (in most cases the level of nucleotide present was below the limits of detection). The n.m.r. experiments were performed on a Bruker AM 500 spectrometer operating at a proton resonance frequency of 500 MHz. For the two-dimensional n.m.r. experiments we used approximately 2·5 mM-enzyme solutions and the sample temperature was usually kept at 300 K. Standard procedures and commercially available software for spectrometer control were used throughout. The residual HDO resonance from samples was suppressed by selective irradiation at the HDO frequency throughout the experiment, except during data acquisition. Quadrature detection and its phase-cycle generated $\omega_1$-analogue in two-dimensional spectroscopy were used in all

† Abbreviations used: AdK, adenylate kinase; n.m.r., nuclear magnetic resonance; D, deuterium; COSY, correlated spectroscopy; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; p.p.m., parts per million.



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experiments. All two-dimensional experiments were done in the phase-sensitive mode. Typical experimental data for the two-dimensional spectra were: COSY-experiment: time-domain matrix size 512 × 4096, sweep-width 5102 Hz in either dimension, relaxation delay 1 second, 256 scans per time increment; frequency-domain matrix 4096 × 4096; an unshifted sine bell filter was applied before transformation in both dimensions; NOESY-experiment: time-domain matrix size 512 × 4096, relaxation delay 1·1 seconds, mixing time 0·15 second, random variation of mixing time 13%; a shifted ($\pi$/32) sine bell filter was applied in both dimensions. (For recent reviews on the two-dimensional methods used, see Wüthrich, 1986; Bax & Lerner, 1986; Ernst *et al.*, 1987.) All the spectra were referenced to internal sodium 4,4-dimethyl-4-silapentanesulfonate.

The one-dimensional $^1$H-n.m.r. spectrum of *E. coli* adenylate kinase in the aromatic region is surprisingly well resolved in the aromatic region (Fig. 1). At pH 6·3 it is pretty easy to discern four singlet resonances in the low-field part of the aromatic region of the *E. coli* protein. These correspond to the C-2 protons from the imidazole rings of the enzyme's three histidyl residues and one imidazole C-4 proton. The broad resonances at 8·3 parts per million originate from amide protons, which in the native protein structure exchange slowly with the solvent deuterium. In addition, there are several clearly resolved doublets of intensity (as calibrated against the histidyl resonances with intensity from one proton) that obviously belong to tyrosyl or phenylalanyl ring spin systems.

The *E. coli* adenylate kinase was found to have a large pH-stability range, either side of physiological pH, and a high capacity for renaturation after pH denaturation. Figure 2 shows spectra taken consecutively in strongly acidic, alkaline, and neutral solution. The n.m.r. spectra indicate that the AdK tertiary structure unfolds below approximately pH 3·5 and above about pH 10·5. The n.m.r. spectrum obtained at pH 6·5, after acidic and subsequent basic denaturation, shows that the protein refolds spontaneously when brought back to neutral pH. It is clear from the identity of the $^1$H-n.m.r. resonances of the aromatic residues before and after acid and base treatment that the refolding is virtually complete. In addition, activity tests before and after incubation of the protein at pH 1 for several minutes gave identical results. The broad resonances at 8·3 p.p.m. disappear during the denaturation and renaturation procedure, probably *via* proton–deuteron exchange; this supports their identification as amide protons which slowly exchange in the native enzyme.

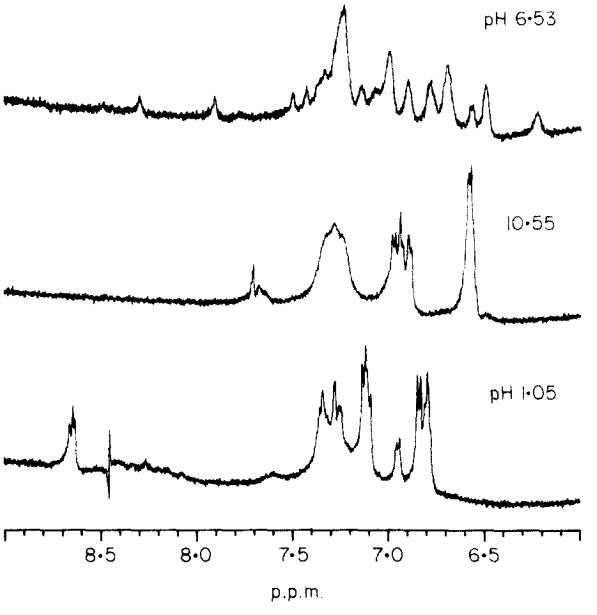


**Figure 2.** pH denaturation of *E. coli* adenylate kinase. Upper spectrum: temperature, 300 K; buffer 50 mM-phosphate; protein concentration, 1 mM; pH 6·5. Middle spectrum: as above, pH 10·5. Lower spectrum: as above, pH 1·0. The spectra were taken subsequently from lower to upper with the same sample.

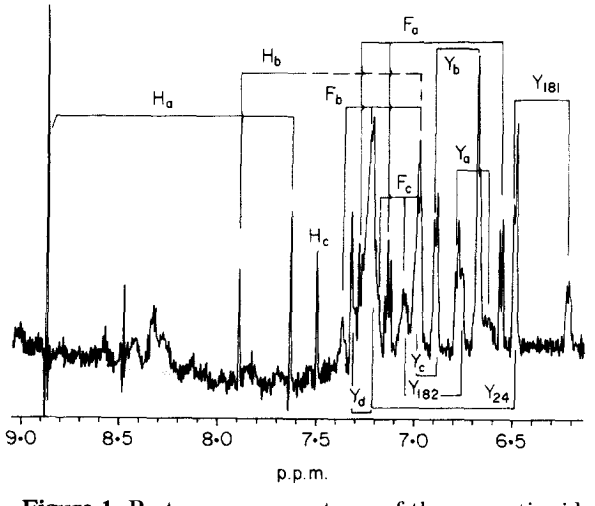


**Figure 1.** Proton n.m.r. spectrum of the aromatic side-chains of *E. coli* adenylate kinase. Temperature, 300 K; buffer, 50 mM-phosphate (pH 6·3); protein concentration, 2·8 mM. The spectrum is digitally processed by Gaussian line sharpening.

The COSY spectrum of *E. coli* adenylate kinase contains several cross-peaks characteristic of His, Phe, and Tyr side-chain spin systems. We could identify all seven tyrosyl ring spin systems and three out of five phenylalanyl ring spin systems unambiguously. In addition, two out of the three imidazole spin systems could also be identified with the chemical shift of the C-2H and C-4H resonances as expected from the one-dimensional spectrum (Fig. 3 and Table 1). The additional singlet resonance from the protein observed in the one-dimensional spectrum therefore originates from the C-2H of the imidazole ring of the remaining His residue. All the aromatic resonances observed lie within the usual chemical shift range expected for

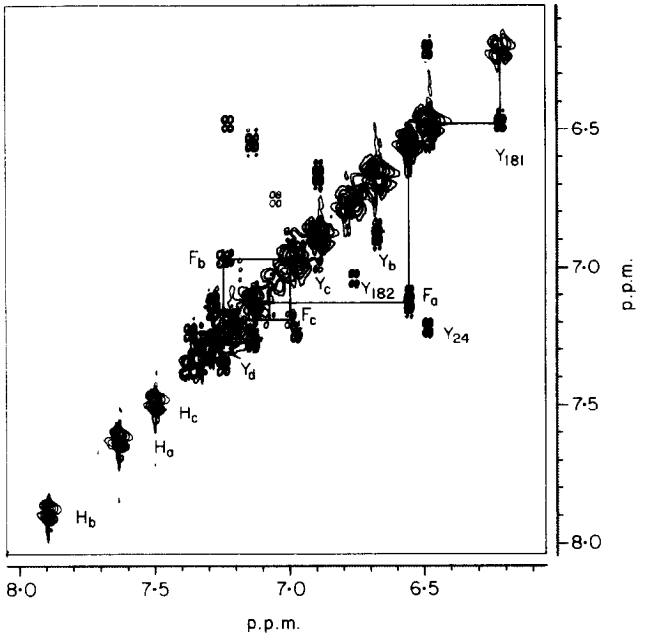


**Figure 3.** COSY spectrum of *E. coli* adenylate kinase. Conditions are as for Fig. 1. Temperature, 300 K; buffer, 50 mM-phosphate (pH 6·3); protein concentration, 2·8 mM.

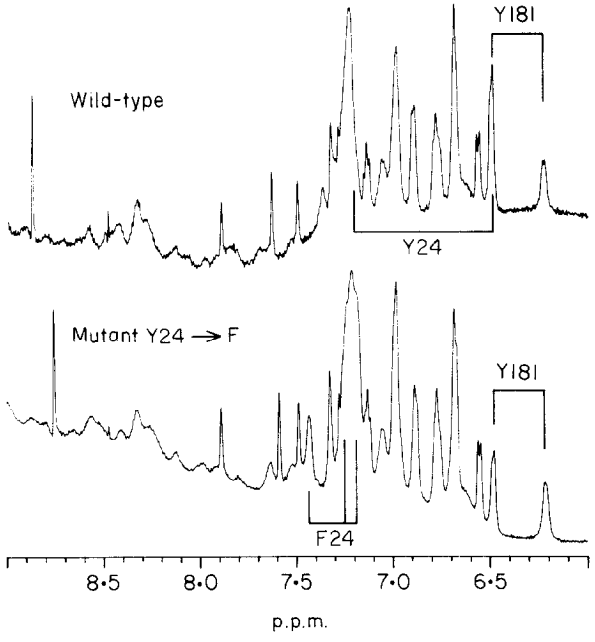


**Figure 4.** Comparison of the aromatic region of the spectrum of wild-type and $Y^{24}$ mutant AdK. Conditions were the same as for Fig. 1. Top: wild-type spectrum. Bottom: $Y^{24}\rightarrow F$ mutant spectrum.

amino acids in proteins at this pH (see, e.g. Wüthrich, 1986; Gross & Kalbitzer, 1988).

In the NOESY spectrum (not shown) we observed a strong cross-peak between two tyrosyl side-chains. The tertiary structure of homologous regions of the porcine AdK suggests that only the side-chains of Tyr181 and Tyr182 are close enough to each other for the generation of a detectable NOE. In addition, their relative location as derived from the AdK structure suggests that the high field resonances belong to Tyr181. The NOESY cross-peaks for the corresponding amino acids from porcine AdK were also observed (Klaus, 1987).

By site-specific mutagenesis of *E. coli* adenylate

**Table 1**

*Chemical shift values of aromatic residues in* E. coli *AdK*

| | | |
|---|---|---|
| Tyr181 | Ortho | 6·48 |
| | Meta | 6·21 |
| Tyr182 | Ortho | 7·05 |
| | Meta | 6·76 |
| Tyr24 | Ortho | 7·22 |
| | Meta | 6·48 |
| Tyr[a] | Ortho | 6·79 |
| | Meta | 6·50 |
| Tyr[b] | Ortho | 6·89 |
| | Meta | 6·68 |
| Tyr[c] | Ortho | 7·00 |
| | Meta | 6·90 |
| Tyr[d] | Ortho | 7·30 |
| | Meta | 7·22 |
| Phe[a] | Meta | 7·13 |
| | Ortho | 7·28 |
| | Para | 6·55 |
| Phe[b] | Meta | 7·24 |
| | Ortho | 7·37 |
| | Para | 6·98 |
| Phe[c] | Meta | 7·20 |
| | Ortho | 7·07 |
| | Para | 7·00 |
| His[a] | C-2H | 8·83 |
| | C-4H | 7·63 |
| His[b] | C-2H | 7·89 |
| | C-4H | 6·98 |
| His[c] | C-2H | 7·50 |
| | C-4H | ? |

ph 6·3; imidazole C-4H of His[c] could not be detected.

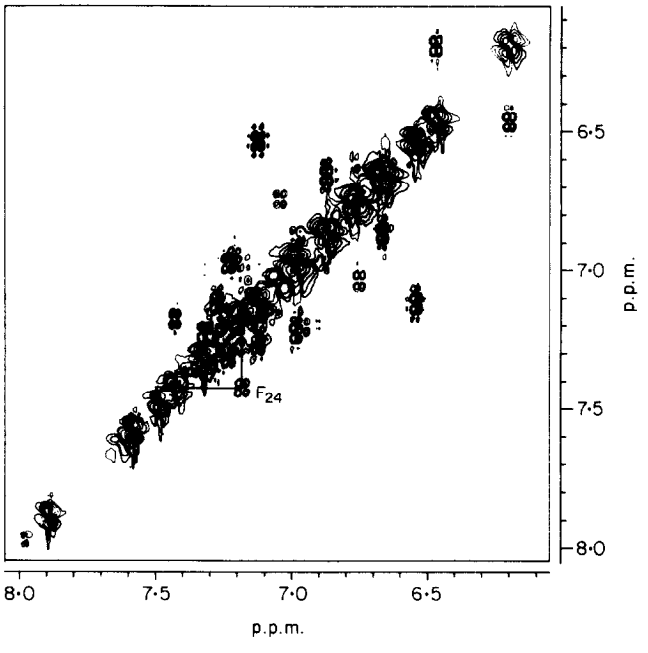


**Figure 5.** COSY spectrum of the aromatic residues of *E. coli* adenylate kinase $Y^{24}\rightarrow F$ mutant. Conditions were the same as for Fig. 1.

kinase (Reinstein *et al.*, unpublished results) we replaced Tyr24 with a phenylalanyl residue. Even in the one-dimensional n.m.r. spectrum the change within the aromatic region could be observed (Fig. 4). In addition, we also recorded a COSY spectrum of the $Y^{24}$ mutant protein. This renders the assignment of the Tyr24 ring spin system obvious, as the corresponding cross-peak is no longer visible in the spectrum of the mutant protein (Fig. 5). Surprisingly, the spectrum of the $Y^{24}$ mutant protein is otherwise only scarcely different from the spectrum of the wild-type protein, showing that the substitution of Tyr with Phe at this position is very conservative. In addition, activity tests showed that there was no difference between wild-type and mutant enzymic activity, which supports a very similar tertiary structure for the mutant protein, as suggested by the virtually identical COSY spectra of the wild-type and the $Y^{24}$-mutant protein.

**Imke Bock**
**Jochen Reinstein**
**Martin Brune**
**Alfred Wittinghofer**
**Paul Rösch**

Max-Planck-Institute for Medical Research
Dept. of Biophysics
Jahnstr. 29
D-6900 Heidelberg 1, Germany

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