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LETTERS TO THE EDITOR

Assignment of Aromatic Spin Systems in the ^{1}H Nuclear Magnetic Resonance Spectrum of Adenylate Kinase

A combination of selective spin decoupling, two-dimensional double quantum spectroscopy, correlated spectroscopy (COSY), and pH titration experiments brought about the assignment of all tyrosyl spin systems and completed the assignment of the histidyl spin systems in porcine adenylate kinase. In the detection of the tyrosyl spin systems it proved to be advantageous to resort to the COSY method rather than to two-dimensional double quantum spectroscopy. In the titration experiments, His189 revealed a second apparent pK value at pH 8·3. which is explained by deprotonation of the adjacent residue Cys187. None of the seven tyrosyl side-chains shows any evidence for deprotonation up to the point of denaturation of the protein, which took place around pH 10.

The X-ray structure of the ATP : AMP phosphotransferase (myokinase, EC 2.7.4.3; Noda, 1973) from porcine muscle is known at 0·3 nm resolution (Schulz *et al.*, 1974); the primary structure (Heil *et al.*, 1974) reveals the presence of two histidyl, five phenylalanyl and seven tyrosyl residues as the only amino acids which are expected to contribute to the aromatic part of the ^{1}H nuclear magnetic resonance spectrum. Of the two histidyl residues, numbers 36 and 189 in the sequence, C-2-H of His36 as well as of His189 in the porcine enzyme have been identified (McDonald *et al.*, 1975); also, a resonance within the bulk of the aromatic part of the spectrum has been assigned to C-4-H of His36 in the human enzyme. This enzyme is virtually identical to the porcine one in terms of primary structure: 185 out of a total of 194 amino acids are identical (von Zabern *et al.*, 1976). The ^{1}H n.m.r.† spectra are also nearly identical (Kalbitzer *et al.*, 1982). Other resonances of the aromatic part of the ^{1}H n.m.r. spectrum have not yet been assigned to types of amino acid residues, let alone to specific residues in the sequence of the protein. So, as a first step towards the understanding of the ^{1}H n.m.r. spectrum, we tried to identify at least some of the aromatic spin systems. The spin systems which are expected to be identified most easily among the aromatic resonances are those composed of the protons of tyrosyl rings.

Porcine adenylate kinase was purified according to Barzu & Michelson (1983) with minor modifications.

n.m.r. measurements were carried out on a Bruker CXP 360 spectrometer equipped with a standard Aspect 2000 data system and a CDC disk storage system. Samples were measured in 10 mm sample tubes (Wilmad, Buena, NJ and Norrell). Standard Bruker disk-based software was used throughout. The sample for the COSY and double quantum spectra was prepared by redissolving freeze-dried protein in 50 mM-Tris · HCl (pH 7·4), 99·75% D_2O, 2 mM-EDTA, 10 mM-1,4-dithioerythritol to a final concentration of about 2·5 mM in a 2-ml volume. Amide protons were pre-exchanged against D_2O at room temperature for several hours. The sample temperature was 298 K unless indicated otherwise. The pH was adjusted by addition of NaOD and DCl. The activity of the protein decreased by about one-third during the two-dimensional measurements. A ^{1}H n.m.r. spectrum taken after the two-dimensional measurements was virtually identical to one taken before the measurements.

Representative parameters for one-dimensional spectra were: 90° flip angle (about 14 μs); 16 K data points; spectral width: 3600 to 4000 Hz; no relaxation delay; HDO resonance decoupled by pre-irradiation; 500 to 1000 scans were accumulated in order to improve the signal-to-noise ratio.

J-decoupling. The sample for the J-decoupling experiments was prepared in a way analogous to those prepared for the two-dimensional experiments. The concentration was 1 mM in a 0·5 ml volume, the measurement performed in a 5 mm sample tube. J-decoupling was performed by the standard gated-decoupling method.

COSY spectra. COSY spectra were acquired using the original pulse sequence (Aue *et al.*, 1976): $(90°\text{-}t_1\text{-}90°\text{-FID}(t_2)\text{-}t_{rel})_n$ with the transmitter phase cycling as suggested by Bax *et al.* (1981*a*) with a superimposed CYCLOPS phasing (Hoult & Richards, 1975). Quadrature detection and its equivalent was used in w_2 and w_1 dimension, respectively (Bax *et al.*, 1981*a*; Wider *et al.*, 1984), with the carrier placed in the center of the spectrum. Sixteen dummy pulse trains were employed before pulse sequences with a new t_1 value were started. The system was allowed to reach equilibrium during a total of two seconds between the second 90° pulse and the subsequent pulse sequence. We used a 256 kw time-space data matrix (1 kw × 256 w), which yielded a 256 kw frequency domain matrix

† Abbreviations used: n.m.r., nuclear magnetic resonance; D, deuterium; p.p.m., parts per million.

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(512 w × 512 w) after zero-filling once in the t_1 direction and Fourier transformation. The sweep width was 3105 Hz in the w_1 dimension as well as in the w_2 dimension, which resulted in a final digital resolution of 6 Hz in either dimension. The free induction decay (FID) in the t_2 direction was collected for 0·116 second, 624 scans were collected for every value of t_1, the final measuring time being approximately 90 hours. The time-domain data matrix was multiplied with a sine bell squared function in the w_2 dimension and a sine bell function in the w_1 dimension (de Marco & Wüthrich, 1976). A symmetrization algorithm with the diagonal from lower left to upper right as symmetry axes was used (Baumann *et al.*, 1981).

Double quantum spectra. Double-quantum spectroscopy in proton n.m.r. (Hore *et al.*, 1982) is an offspring of the INADEQUATE method in ^{13}C n.m.r., which was originally developed in order to simplify ^{13}C n.m.r. spectra by suppression of signal from ^{13}C nuclei without directly coupled ^{13}C partners (Bax *et al.*, 1980). Double quantum spectroscopy offers the advantage over COSY that strong diagonal peaks are missing, thus connectivities between very closely spaced resonances may be detected.

For the detection of double quantum spectra the original INADEQUATE pulse sequence was used as described by Bax *et al.* (1980): (90°-t-180°-t-90°-t_1-90°-FID(t_2)-t_{rel})$_n$, t being a $1/(4nJ)$ delay. We used a delay $t = 1/(4J)$ for $J = 8$ Hz. The phase cycling scheme as described by Bax *et al.* (1980) with superimposed CYCLOPS phase cycling (Hoult & Richards, 1975) was employed. Quadrature detection and its equivalent was used in w_2 and w_1 dimension, respectively, which modified the above basic pulse sequence according to a pulse scheme with a 64-step repeat as described by Bax *et al.* (1981*b*). The carrier frequency was placed in the middle of the spectrum. The relaxation time between the last pulse and the first pulse of the next experiment was chosen to be two seconds, each FID was collected for 0·116 second, 640 scans were averaged for each value of t_1, which resulted in a total measuring time of 94 hours. The sweep width was 3105 Hz in the w_2 dimension and 6210 Hz in the w_1 dimension. The time domain matrix $s(t_1, t_2)$ of size 256 kw (1 kw × 256 w) was developed on a frequency domain matrix of size 256 kw (512 w × 512 w) after zero-filling once in the w_1 dimension. This resulted in a final digital resolution of 6 Hz in the w_2 dimension and 12 Hz in the w_1 dimension. For signal-to-noise improvement a sine bell multiplication was performed in the w_1 direction and a sine bell squared multiplication in the w_2 direction.

Figure 1 shows the ^1H n.m.r. spectrum of adenylate kinase. The histidyl resonance assignments in the Figure are adopted from McDonald *et al.* (1975) and Kalbitzer *et al.* (1982). The other resonances represent a total of 54 protons, 28 tyrosyl protons, 25 phenylalanyl protons, and the C-4 proton of His189. Only connections in three out of the seven tyrosyl and five phenylalanyl residues could be found by extensive J-decoupling experiments within the aromatic region. This is not extremely surprising in light of the fact that selective spin decoupling in regions with such severe resonance overlap has previously proved impossible in similar cases.

Double quantum measurements. The demonstration that double quantum spectroscopy could be applied for the assignment of resonances to spin systems of aromatic side-chains of amino acids (Wagner & Zuiderweg, 1983) led us to try resonance assignments with this method in the adenylate

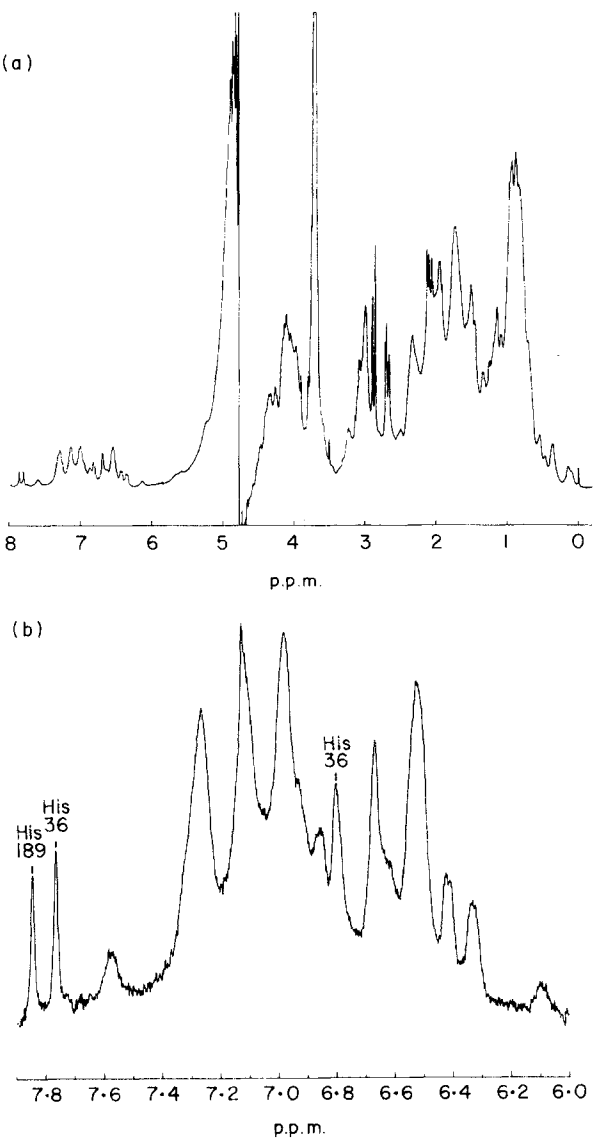

Figure 1. (a) The ^1H n.m.r. spectrum of adenylate kinase. The strong resonances between 2·7 p.p.m. and 4·8 p.p.m. are due to HDO and 1,4-dithioerythritol. (b) The low-field part of the H n.m.r. spectrum of porcine adenylate kinase. The spectrum represents the aromatic protons of a total of 14 amino acids, i.e. 7 Tyr, 5 Phe and 2 His residues. The resonances of the C-2-H of His36 as well as His189 and the C-4-H of His36 have been assigned (McDonald *et al.*, 1975; Kalbitzer *et al.*, 1982).

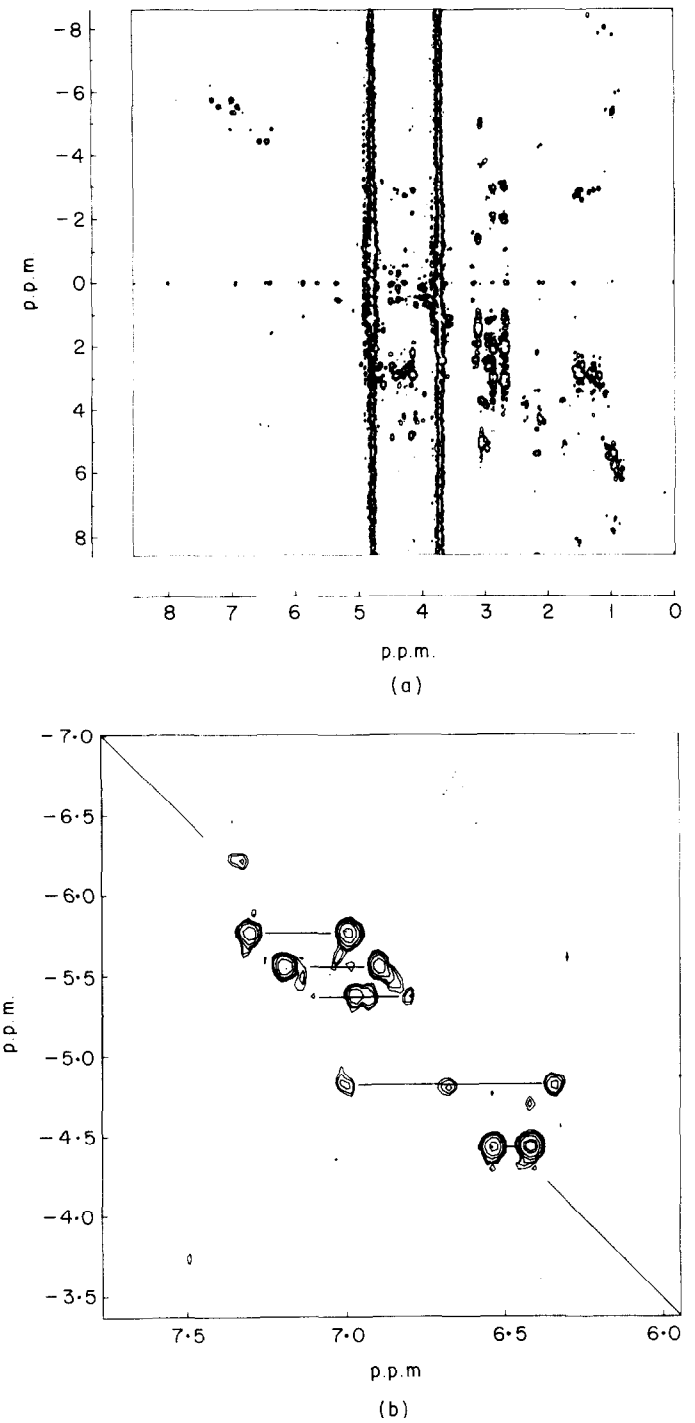

Figure 2. (a) Complete double quantum spectrum of porcine adenylate kinase. The double quantum axes runs from lower right to upper left. Chemical shifts are given as p.p.m. from 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) (w_2 axes) and p.p.m. from the carrier frequency (w_1 axes). The 2 intense peaks in the spectral center are due to HDO and Tris buffer. (b) Same as (a), resonances of aromatic protons only.

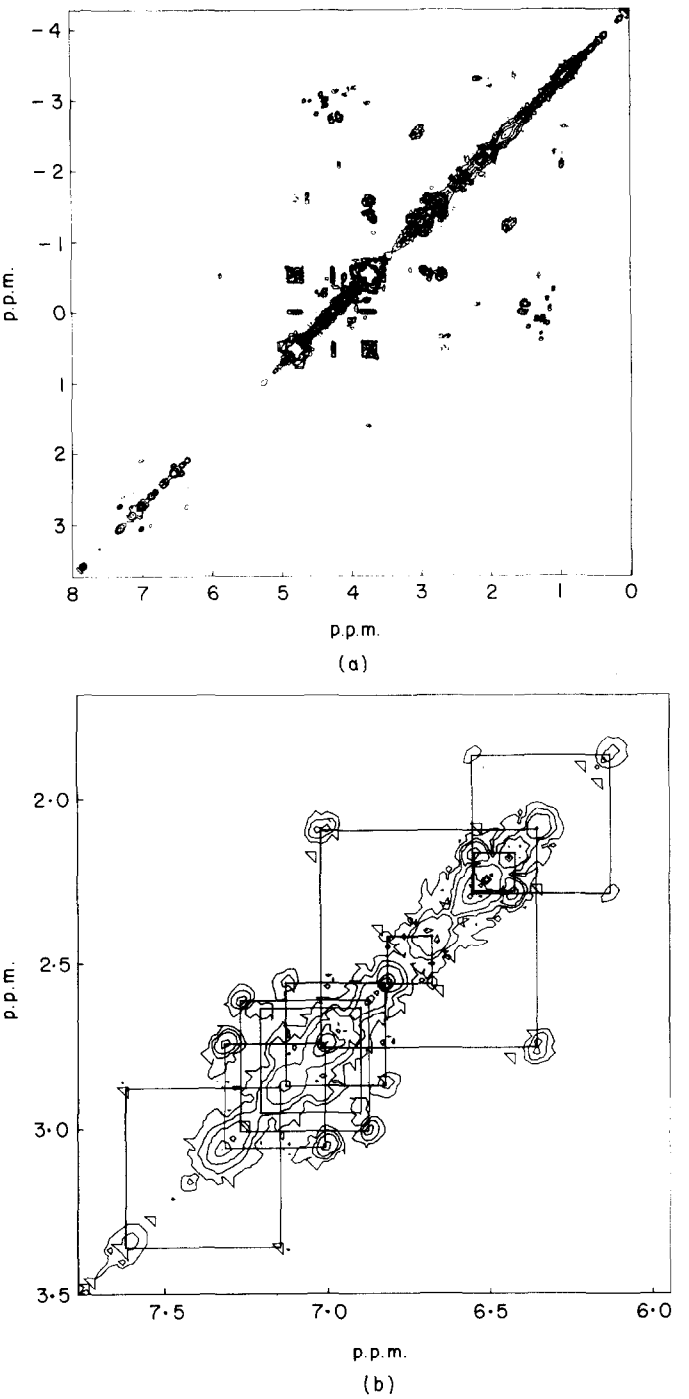

Figure 3. (a) Complete COSY spectrum of porcine adenylate kinase. The symmetry axes runs from lower left to upper right. Chemical shifts are given as p.p.m. from 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) (w_2 axes) and p.p.m. from the carrier frequency (w_1 axes). (b) Same as (a), resonances of aromatic protons only.

kinase spectrum. Figure 2(a) shows the total double quantum spectrum of adenylate kinase, Figure 2(b) the aromatic part thereof, the double quantum diagonal running from lower right to upper left. Indicated in Figure 2(b) are connectivities among five different spin systems, which may be identified as tyrosyl side-chain resonances according to Boyd *et al.* (1983). Other connectivities could not be detected in this part of the spectrum, in particular those from the remaining two tyrosyl residues were still missing. Therefore, we resorted to the

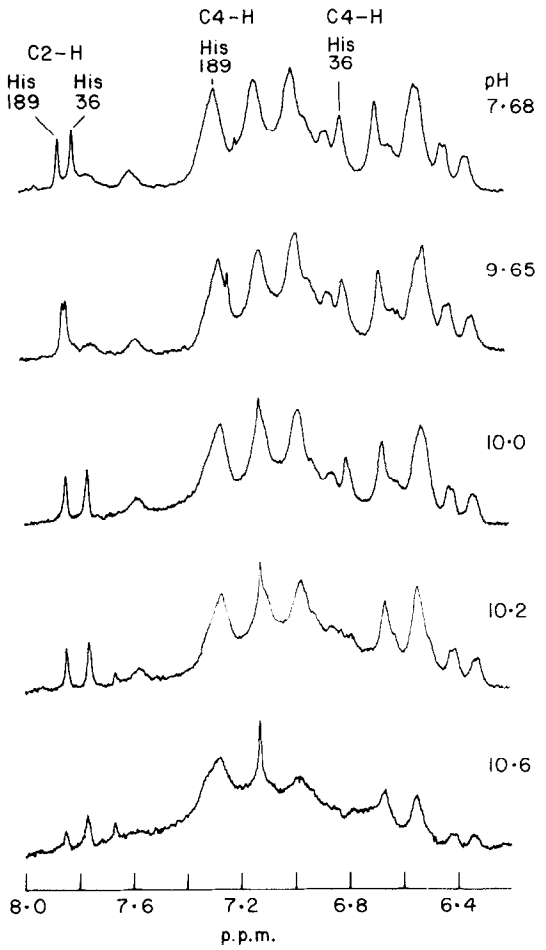

Figure 4. Resonances of the aromatic protons of adenylate kinase at different pH values.

other two-dimensional method which is supposed to give the same type of information as double quantum spectroscopy, namely COSY.

COSY measurements. The total transformed and symmetrized 1 k × 256*w* data matrix is shown in Figure 3(a) in the form of a contour plot. A closer inspection of the aromatic spectral part, shown in Figure 3(b), shows that it is clearly possible to assign the spin systems, at least in part, belonging to the aromatic part of nine residues.

pH titrations. We performed a pH titration starting from neutral pH values which would cover the p*K* region of free tyrosine (p*K*, 10·1). Figure 4 shows the aromatic spectral part at a few pH values in this region. Clearly, virtually the only major change in the spectrum up to pH 9·5 is a shift of the C-2-H of His189 to high field, paralleled by the shift of a singlet resonance starting from 7·3 p.p.m. at pH 7. Determination of the apparent p*K* value by a least-squares routine yields similar p*K* values for this singlet resonance and for the the C-2-H of His189, thus rendering the assignment of this singlet resonance to the C-4-H of His189 and completing the assignment of the histidyl resonances. In addition, a small shift of the C-2-H of His36 to higher p.p.m. values on raising the pH value is

Table 1

Titration parameters for the histidyl residues

His36	S_{AH}	S_{A^-}	S'_{A^-}
C-2-H	(8·69)	(7·75) 7·80	7·84
C-4-H	(7·01)	(6·79) 6·80)	n.a.
His189			
C-2-H	(7·99)	(7·84) 7·86	7·76
C-4-H	n.a.	7·28	7·13

Numbers in parentheses are for the human enzyme (Kalbitzer *et al.*, 1982). n.a., not available.

observed. The titration parameters for the histidyl residues are compiled in Table 1. No shift of any of the tyrosyl resonances in the aromatic region was observed up to about pH 10. At this pH value denaturation of the protein starts. This is most clearly seen by the appearance of a new peak at 7·65 p.p.m., which is accompanied by the decrease of the C-2-H resonance of His36. This new peak is close to the random coil position of the C-2-H of the histidyl residue in a model tetrapeptide (Bundi & Wüthrich, 1979) and is therefore ascribed to the C-2-H of His36 in the denatured protein under slow exchange conditions on the n.m.r. timescale. The exchange rate between the native and the denatured form of the protein is less than 400 s^{-1}, according to the difference in chemical shift between the two resonances originating from the C-2-H of His36. Also, the resonance assigned to the C-4-H of His36 decreases in intensity, probably contributing to the new resonance at about 7·15 p.p.m., the approximate random coil position of the C-4-H of histidyl residues. Other resonances in the aromatic part also change intensities as a consequence of the denaturation of the protein.

The aliphatic part of the spectrum did not show any changes prior to the point where denaturation was indicated by the appearance of the second resonance of the C-2-H of His36.

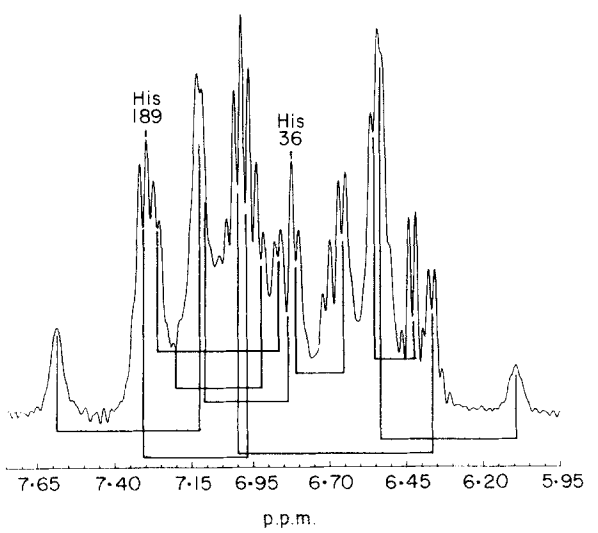

Figure 5. Assignment of resonances of aromatic protons of porcine adenylate kinase as described by McDonald *et al.* (1975), Kalbitzer *et al.* (1982), and this work.

Table 2
Results of the assignment procedures

Tyrosine	Chemical shift		Identified by
A	6·35	7·01	COSY, DQ, *J*-decoupling
B	6·42	6·54	COSY, DQ, *J*-decoupling
C	6·67	6·82	COSY
D	6·81	7·12	COSY, DQ
E	6·87	7·26	COSY
F	6·89	7·20	COSY, DQ
G	7·00	7·31	COSY, DQ, *J*-decoupling
Phenylalanine			
A	6·13	6·55	COSY
E	7·14	7·62	COSY

DQ, double quantum spectroscopy.

In order to decide which of the nine spin systems identified in the COSY map really represent tyrosyl spin systems, we took a one-dimensional spectrum at 308 K, a temperature at which the protein has a half-life in the order of hours at best. A subsequent Gaussian line-sharpening routine (Ferrige & Lindon, 1978) yielded the spectrum shown in Figure 5. Clearly, most of the nine connectivities end in a doublet-like structure, as expected for resonances originating from protons in tyrosyl rings undergoing rapid ring-flipping motion, whereas the resonances at high and at low field do not show any multiplet structure. This leads us to the result that the seven central connectivities indicated in the Figure represent the ring protons of the seven tyrosyl residues, whereas the other two connectivities represent the ring protons of phenylalanyl residues shifted to high and low field, respectively, from the main body of the aromatic resonances.

Table 2 lists the results of the assignment procedures together with an indication of the kind of evidence for these assignments, i.e. double quantum spectroscopy, COSY or *J*-decoupling.

The experiments described in this letter have led so far to the assignment of the aromatic part of all seven tyrosyl spin systems and two phenylalanyl spin systems in porcine adenylate kinase, leaving three phenylalanyl spin systems to be assigned; as a by-product, the C-4-H of His189 has been identified, so that now the imidazole part of the two histidines is assigned completely. Ongoing experiments aim at specifically nitrating the tyrosyl rings and therewith assigning tyrosyl resonances to their position in the primary structure.

Although the pH titrations did not reveal any pK values of the tyrosyl residues, they lead to the assignment of the C-4-H of His189. The apparent second pK value of this residue at pH 8·3 is most probably caused by the deprotonation of a nearby amino acid residue. The pK_a value of 8·3 suggests a cysteinyl residue. In fact, the amino acid sequence of the protein shows a cysteine at position 187. According to the crystal structure, His189 and Cys187 are residues on the surface of adenylate kinase, so that the average distance between these two residues could easily be even less in solution than suggested by the crystal structure. In any case, the presence of Cys187 in the vicinity of His189 explains the second apparent pK value of the latter residue satisfactorily.

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