NMR Studies of Protein-Nucleotide Interactions: p21 and Adenylate Kinase

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Abstract

We performed one- and two-dimensional nuclear magnetic resonance (NMR) studies, in particular substrate-protein nuclear Overhauser effect (NOESY) measurements, as well as nucleotide and P^1,P^5-bis(5'-adenosine)-pentaphosphate (AP_5A) titrations of the E. coli adenylate kinase. Also, the same types of experiment were performed with the H-ras oncogene product p21. These experiments led us to the following conclusions:

1. $AP_5A{\cdot}Mg^{2+}$ binds to both nucleotide sites simultaneously and thus simulates the active complex.
2. The $ATP{\cdot}Mg^{2+}$ adenine in the $AK_{EC}{\cdot}AP_5A{\cdot}Mg^{2+}$ complex is located close to His^{134} and Phe^{19}, i. e the $ATP{\cdot}Mg^{2+}$ site in solution is identical to the $ATP{\cdot}Mg^{2+}$ site in the crystal (Müller, C. W., & Schulz, G. E. (1988) *J. Mol. Biol. 202*, 909-912).
3. The AK_{EC} 'G-loop' with bound $ATP{\cdot}Mg^{2+}$ is structurally highly homologous to the loop region in the oncogene product p21 with bound $GTP{\cdot}Mg^{2+}$ in solution as well as in the crystal (Pai, E. F., Kabsch, W., John, J., Holmes, K. C., & Wittinghofer, A. (1989) *Nature*, in press).

Introduction

Adenylate kinases (AK, ATP:AMP phosphotransferase, EC 2.7.3.4) catalyse the transfer of the terminal phosphoryl group of ATP to AMP in the presence of a divalent metal ion, physiologically Mg^{2+}, which binds to the triphosphate and one of the product diphosphates, respectivly (Noda, 1971):

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

The enzyme has two different nucleotide binding sites, the AMP-site and the $ATP{\cdot}Mg^{2+}$-site, corresponding to the product ADP and $ADP{\cdot}Mg^{2+}$-site, respectively. The location of these two sites is a subject of much controversy between X-ray crystallographers (Pai et al., 1977; Egner et al., 1987; Müller & Schulz, 1988) and NMR-spectroscopists, (Hamada et al., 1979; Smith & Mildvan, 1982; Fry et al., 1985; Mildvan, 1989). Many of the NMR results, which were obtained with mammalian enzymes exclusively, point to a location of the ATP site close to the His^{36} region (porcine sequence numbering system), whereas the X-ray results, obtained with the E. coli and the yeast enzymes, point to a location of the $ATP{\cdot}Mg^{2+}$ site close to the His^{134} region (E. coli sequence numbering system). The two regions are in completely different parts of the three-dimensional structure and are not homologous, implying a sharp contrast between the X-ray structure and the NMR results.

The bisubstrate analog P^1,P^5-bis(5'-adenosine)-pentaphosphate (AP_5A) is a potent inhibitor of all tested adenylate kinases, in particular of the porcine enzyme (Lienhard and Secemski, 1973; Feldhaus et al., 1975). Whereas it was not possible so far to cocrystallise any of the mammalian adenylate kinases with either one of the substrates or, alternatively, the bisubstrate analog AP_5A, the yeast and the E. coli enzymes cocrystallise with AP_5A (Egner et al., 1986; Müller & Schulz, 1988).

In order to further clarify the location of the $ATP{\cdot}Mg^{2+}$ site and resolve the discrepancy between the differing NMR-results and the X-ray results we performed experiments similar to those we did with the porcine adenylate kinase (Rösch et al., 1989) with the E. coli enzyme (molecular weight 23 kDa). Suggestions for the sequence specific assignment for several resonances in the aromatic side chain region of the spectrum were reported earlier (Bock et al., 1988). The E. coli enzyme and the Paracoccus denitrificans sequences are unique among the known adenylate kinase sequences in so far as they contain no His residues homologous to His^{36} in the porcine enzyme; instead, a Gln residue is found in the homologous position (Gln^{28} in E. coli AK (Haase et. al., 1988); Gln^{29} in Paracoccus denitrificans AK (Spürgin et al., 1989)).

The H-ras oncogene product p21 is a GTP-hydrolase with a molecular weight of 21 kDa. It is supposed to be involved in regulatory processes of cellular growth and development. p21 is usually purified as p21·GDP·Mg^{2+}, since the dissociation constant of this complex is very low (John et al., 1988). The X-ray structure was recently solved by Pai et al. (1989).

From studies of sequence homologies it was suggested that a region close to the N-terminus adopts a very similar conformation for adenylate kinases and p21 (Möller & Amons, 1985). It was suggested that this glycine-rich loop (G-loop; Dreusicke & Schulz, 1988) is involved in phosphate and/or metal binding. Thus, it was interesting to see whether the relation of this region to the bound nucleoside triphosphate is similar in p21 and adenylate kinases.

Materials and Methods

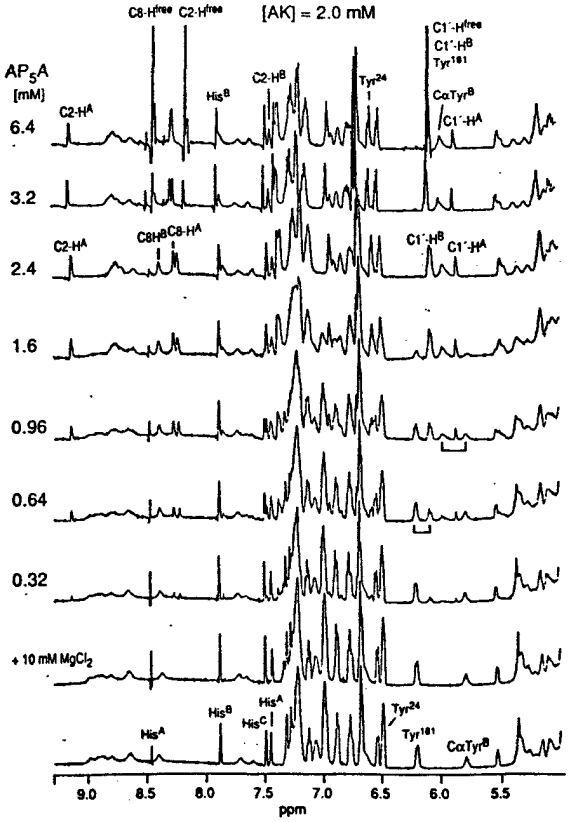
The standard protein preparations for p21 and adenylate kinase have been used. Also, standard NMR spectroscopic techniques and standard procedures were used for all experiments. For more details, see Vetter et al. (1989) and Schlichting et al. (1989).

Results

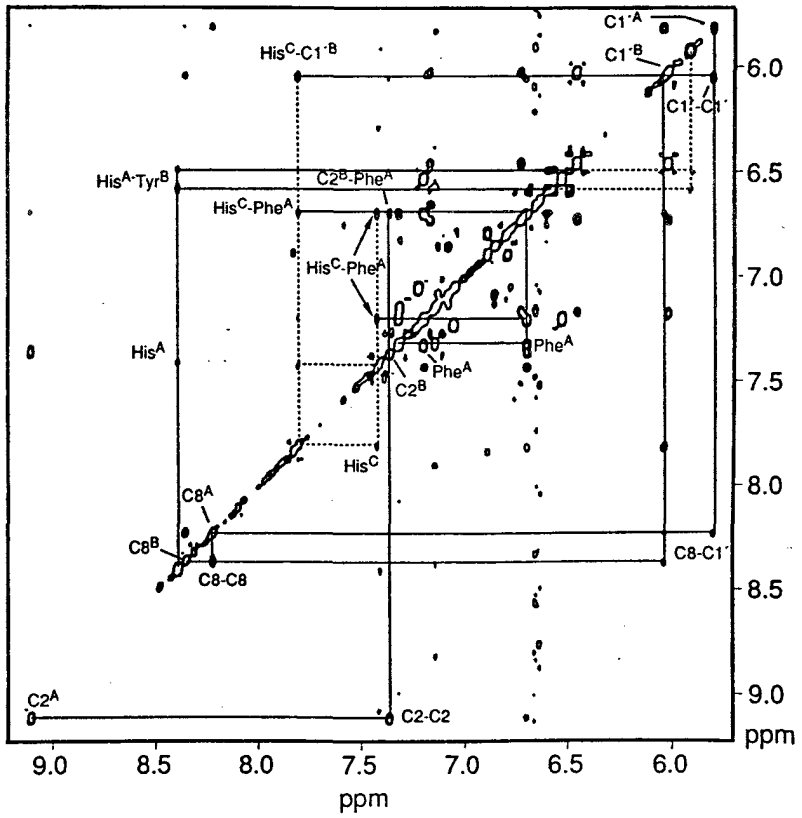
The AK_{EC}·AP_5A·Mg^{2+} complex:
Titration of AK_{EC} with AP_5A in the presence of $MgCl_2$ leads to the appearance of two sets of resonances from bound AP_5A, corresponding to resonances from the adenine C2-H, the adenine C8-H, and the ribose C1'-H from both adenosines (fig. 1). One of the resonances of bound AP_5A·Mg^{2+} exhibits a chemical shift close to the value determined for the C2-H resonances of site A of porcine AK_1 bound AP_5A·Mg^{2+} as reported earlier (Rösch et al., 1989). The other C2-H resonance of bound AP_5A·Mg^{2+} can be located easily by a 1D saturation transfer experiment, which shows a decrease of the second C2-H resonance on irradiation of the low-field C2-H resonance due to chemical exchange, as the AP_5A·Mg^{2+} may dissociate from the enzyme and bind again with the base moieties reversed. Most protein resonances are in slow exchange during the titration, so that almost none of them can be followed with certainty. Clearcut changes of the protein on complex formation include the downfield shift of the C3/5-H resonance of Tyr^{24}, the upfield shift of the resonance assigned to the C3/5-H of Tyr^{181} (Bock et al., 1988), and the downfield shift of the resonance at 5.8 ppm suggested to represent the backbone C_α-H of Tyr^B as indicated by the NOESY crosspeak between the corresponding ring proton resonances and this resonance.

NOESY experiments:
In the aromatic amino acid resonance region of the 2D NOESY spectrum of the AK_{EC}·AP_5A·Mg^{2+} complex several clearcut intramolecular cross-peaks can be observed (fig. 2). For the AP_5A molecule these are in particular the exchange cross-peaks between the two C2-H resonances and the NOE cross-peaks between the ribose C1'-H resonance and the corresponding base C8-H resonance of either site. In addition, an intraprotein cross peak between the His^C imidazole C2-H resonance and the resonances of the phenyl ring of the Phe^A residue (Bock et. al., 1988) can be observed. From a comparison with the X-ray structure it can be inferred immediately that these resonances represent His^{134} and Phe^{19}, respectively, since this is the only pair of this type of residues with a mutual distance of less than 0.5 nm in the crystal structure of the AK_{EC}·AP_5A·Mg^{2+} complex (Müller & Schulz, 1988) and thus is the only pair of this type which is expected to generate cross peaks in the NOESY spectrum. By the same argument the residue originally labeled His^A is identified as His^{172} and the residue originally labeled Tyr^B is identified as Tyr^{171}, since His^A and Tyr^B show a cross peak in the NOESY spectrum and the pair His^{172}-Tyr^{171} is the only one of this type with a side chain to side chain distance of less than 0.5 nm. His^B is then identified as His^{126} by exclusion.

Two prominent substrate-protein intermolecular cross-peaks appear in the aromatic spectral region: first, between the high-field adenine C2-H resonance and the high-field resonance of the Phe^{19} aromatic ring protons; secondly, between the low-field resonance of the imidazole ring of His^{134} (it cannot be decided from the present data whether this represents C2-H or C4-H) and the ribose C1'-H resonance belonging to the same site as the high-field adenine C2-H resonance. From these cross-peaks it follows clearly that this base moiety in the AK_{EC}·AP_5A·Mg^{2+} complex is located very close (<.4 nm) to the side chains of residues 134 and 19.



1.) The aromatic ring proton resonance region of the spectrum of AK_{EC} and the $AK_{EC}{\cdot}AP_5A{\cdot}Mg^{2+}$ complex at concentrations of AP_5A as indicated. $[AK_{EC}]$= 2.0 mM; $[MgCl_2]$= 10 mM; HEPES buffer, 50 mM, pH 8.0.



2.) The aromatic ring proton resonance region of the NOESY spectrum of the $AK_{EC}{\cdot}AP_5A{\cdot}Mg^{2+}$ complex. $[AK_{EC}]$= $[AP_5A]$= 1.5 mM; $[MgCl_2]$= 10 mM; HEPES buffer, 50 mM, pH 8.0.

The AK_{EC}·AMP complex:
Complex formation of AMP and E. coli adenylate kinase in the absence of divalent metal ions is accompanied by severe changes of the NMR-spectrum of the protein, exemplified by the aromatic spectral part in fig. 3. All resonances in the aromatic part, including the adenine C2-H, adenine C8-H, and ribose C1'-H resonances of the nucleotide, are in fast exchange between the free and bound forms. From the concentration dependence of the chemical shift of the adenine C2-H and C8-H it can be seen immediately that the C2-H resonance in the fully bound state is shifted to the low-field side of the C8-H resonance, thus reversing the order of chemical shifts in the free nucleotide. Extrapolation of the chemical shift of the C2-H to the fully bound state yields approximately δ_b= 8.82 ppm (see table). This suggests immediatly assignment of the low-field C2-H resonance of the AK_{EC}·AP_5A·Mg^{2+} complex to the AMP-site adenine C2-H.

The AK_{EC}·ATP complex:
Titration of AK_{EC} with ATP in the absence of Mg^{2+} ions resulted in strong distortions of the protein spectrum as well as the nucleotide spectrum, as exemplified by the spectral region between 5.9 and 8.8 ppm in fig. 4. The most remarkable feature of these spectra is the behaviour of the adenine C2-H resonance of ATP, which is shifted to approximately 7.8 ppm at a ATP to protein ratio of about 0.28. The chemical shift of the C2-H resonance can be extrapolated from the chemical shift values measured above this nucleotide to protein ratio and the dissociation constant of the complex (K_d= 40 1M) to be d_b= 7.80 ppm in the bound state (see table). This yields immediately the assignment of the high-field adenine C2-H resonance of the AK_{EC}·AP_5A·Mg^{2+} complex to the ATP-site adenine C2-H, thus corroborating the assignment of the low-field C2-H resonance of this complex to the AMP-site.

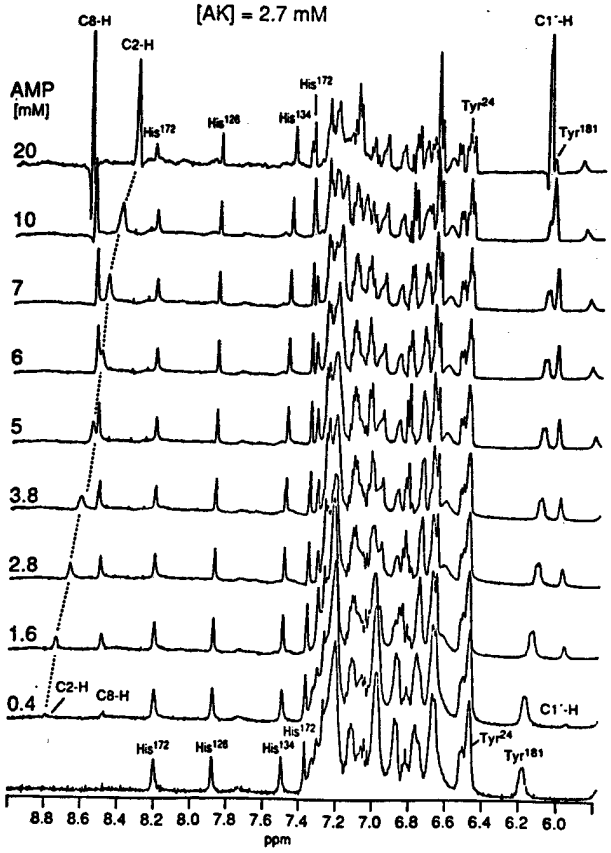
Several resonances of the NMR spectrum of the aromatic side chains of the p21·GDP·Mg^{2+} complex were identified earlier (Schlichting et al., 1988). In addition, we were able to perform sequence specific assignments of aromatic side chain resonances by studies of mutants (Schlichting et al., 1989). This resulted in particular in the assignment of Phe^{28}. In the recently published structure of the p21.nucleotide complex (Pai et al., 1989), Phe^{28} is located close to the ribose ring of the bound nucleotide. This could also be inferred from an earlier X-ray structure of the p21·GDP complex (deVos et al., 1989), although the chain tracing in this structure was shown to be incorrect later (Pai et al., 1989).

The NOESY spectrum of the aromatic side chain protons, the C8-H and the ribose C1'-H shows clear cross peaks between the resonances assigned to the ring protons of Phe^{28} and the C1'-H resonance of the ribose moiety (fig. 5). It thus seems clear that the location of the nucleotide in the crystal is identical to the location of the nucleotide in solution.

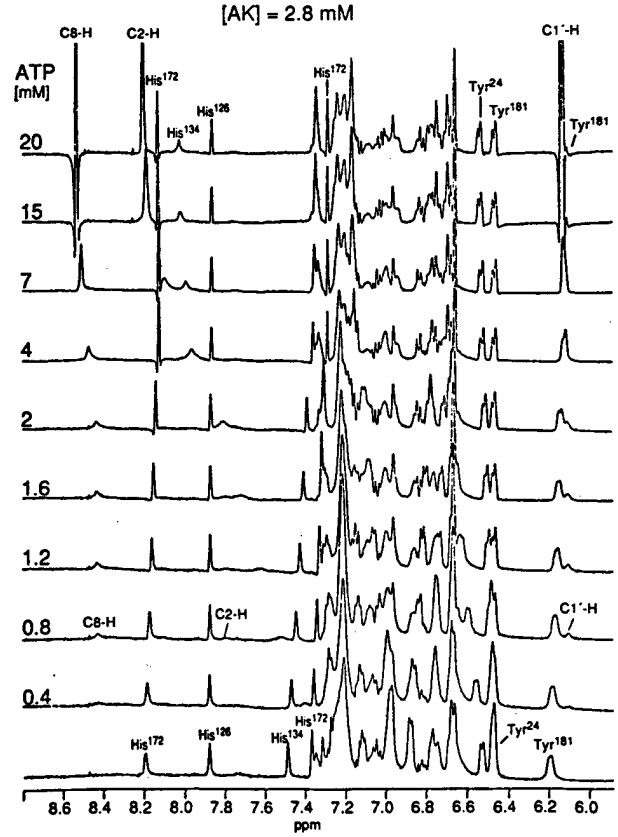
Discussion

From our present studies, it is clear that both adenine moieties of AP_5A bind to the protein in the AK_{EC}·AP_5A·Mg^{2+} complex, a fact that has been treated with care in the past (Egner et al., 1987). Moreover, we conclude from the closeness of the extremely unusual chemical shifts of the C2-protons of bound AP_5A·Mg^{2+} with the shifts of the respective protons of AMP and ATP that the two adenine moieties of the bound AP_5A·Mg^{2+} bind to the two nucleotide sites of the enzyme (see table). Accordingly, the low-field C2-H resonance represents the AMP-site adenine proton, whereas the high-field C2-H resonance represents the corresponding ATP·Mg^{2+}-site proton of the bound AP_5A·Mg^{2+} complex. From the above two-dimensional NOESY results it thus follows clearly that the location of the ATP·Mg^{2+}-site in E. coli adenylate kinase in solution is close to His^{134} and Phe^{19}. It is thus the same location as one of the nucleotide sites observed in the AK_{EC}·AP_5A·Mg^{2+} and AK_Y·AP_5A·Mg^{2+} crystals, respectively (Egner et al., 1987; Müller & Schulz, 1988).

Our earlier experiments with the nucleotide and AP_5A complexes of porcine adenylate kinase did not result in such clear cut assignments, since we were not able to determine the chemical shift of the C2-H of ATP·Mg^{2+} in the fully or even partially bound state. These earlier experiments thus still left open the possibility that the downfield shifted base C2-H resonance originating from the AK_1·AP_5A·Mg^{2+} complex corresponds to the ATP·Mg^{2+}-site adenine resonance. This was considered to be a remote possibility only, since this C2-H resonance of the AK_1·AP_5A·Mg^{2+} complex



3.) The aromatic ring proton resonance region of the spectrum of AK_{EC} and the AK_{EC}·AMP complex at concentrations of AMP as indicated. [AK_{EC}]= 2.7 mM; HEPES buffer, 50 mM, pH 8.0.



4.) The aromatic ring proton resonance region of the spectrum of AK_{EC} and the AK_{EC}·ATP complex at concentrations of ATP as indicated. [AK_{EC}]= 2.8 mM; HEPES buffer, 50 mM, pH 8.0.

and the corresponding proton of the $AK_1 \cdot AMP$ complex displayed chemical shift values which were extraordinarily low-field and very similar to each other (see table). Anyhow, comparison of our present experiments with the experiments on the $AP_5A \cdot Mg^{2+}$ complex of porcine adenylate kinase strengthens our earlier case.

Since we were not able to observe any NOE in the single substrate complexes ($AK \cdot AMP$, $AK \cdot ATP$, or $AK \cdot ATP \cdot Mg^{2+}$) neither with the porcine nor with the E. coli adenylate kinase, we conclude that the combination of correlation time and distance effects allows observation only in the active complex of enzyme and nucleotide as mimicked by the $AK_{EC} \cdot AP_5A \cdot Mg^{2+}$ complex. In earlier papers (reviewed in Mildvan, 1989) this statement was corroborated for the AMP complexes and the NOE was only observed with the $ATP \cdot Mg^{2+}$ complex in the presence of a fivefold to tenfold excess of nucleotide.

The determination of the $ATP \cdot Mg^{2+}$-site in AK_{EC} has another very important consequence: Comparison of the 'G-loop' region of $AK_{EC} \cdot AP_5A \cdot Mg^{2+}$ (Müller & Schulz, 1988) or $AK_Y \cdot AP_5A \cdot Mg^{2+}$ (Egner et al., 1987) with the $ATP \cdot Mg^{2+}$-site as suggested by us shows extreme structural homology to the corresponding loop region of the oncogene product $p21 \cdot GTP \cdot Mg^{2+}$ complex as determined recently (Pai et al., 1989), i.e. the peptide chain structure of the first approximately 23 amino acid residues of either protein and the relative location of the nucleotide triphosphate is virtually identical. This could mean that the structural motif involved in the abstraction of the terminal phosphoryl group from the nucleoside triphosphate is identical in both proteins, thus giving rise to a very similar phosphoryl transfer mechanism. This is in accord with the observation that this sequence motif appears in many nucleotide triphosphate hydrolysing proteins (Möller and Amons, 1983).

Although the $ATP \cdot Mg^{2+}$-site is now clearly defined in the E. coli and in the yeast adenylate kinase (Müller & Schulz, 1989), the location of the AMP-site in these complexes remains a mystery. The location of one of the nucleotide sites close to His^{36} in the porcine $AK_1 \cdot AP_5A \cdot Mg^{2+}$ complex is clearly not supported by the crystallographic results with the E. coli and yeast enzyme complexes (Egner et al., 1987; Müller & Schulz, 1988). Thus, the final undisputable localisation of the AMP-site in the adenylate kinases will have to wait for further NMR and crystallographic studies, both of which are currently under way.

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We would like to thank Marija Isakov and Birgit Brandmeier for skilled technical assistance, Prof. Georg Schulz, Freiburg, for supplying us with the crystallographic coordinates of the $AK_{EC} \cdot AP_5A \cdot Mg^{2+}$ complex and Prof. Kenneth C. Holmes for constant support and encouragement.

Table

Chemical shift of the nucleotide base proton resonances

Compound		C2H	C8H
AP_5A free		8.12	8.44
$AK_1 \cdot AP_5A \cdot Mg^{2+}$	A	8.90	8.40
	B	8.03	8.37
$AK_{EC} \cdot AP_5A \cdot Mg^{2+}$	A	9.14	8.26
	B	7.42	8.39
ATP free		8.26	8.55
$AK_1 \cdot ATP \cdot Mg^{2+}$.		ca. 8.4	ca. 8.5
$AK_{EC} \cdot ATP$		7.80	8.42
AMP free		8.22	8.59
$AK_1 \cdot AMP$		8.65	8.58
$AK_{EC} \cdot AMP$		8.82	8.47

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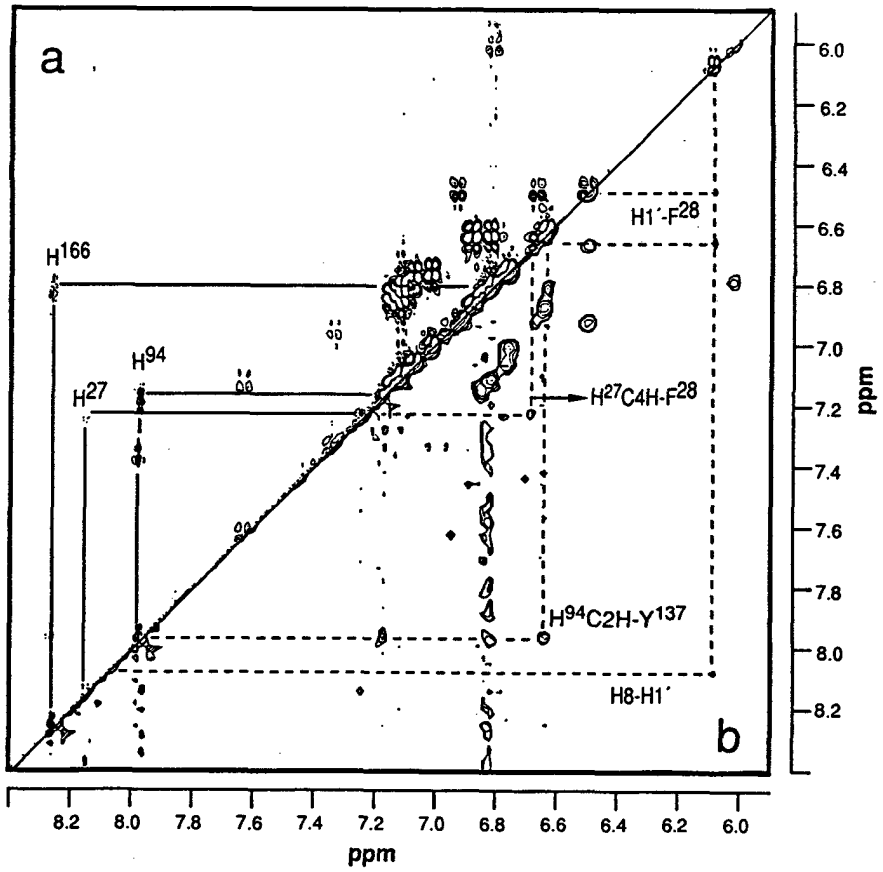

5.) COSY (upper triangle) and NOESY (lower triangle) spectra of the aromatic side chains and the guanine C8-H and ribose C1'-H of the H-ras complex. [p21·GDP·Mg^{2+}]= 3.5 mM; [$MgCl_2$]= 45 mM; [DTE]= 8 mM; [NaN_3]= 4 mM; phosphate buffer, 45 mM, pH 6.6.

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