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Identification of valine/leucine/isoleucine and threonine/alanine/glycine proton-spin systems of *Escherichia coli* adenylate kinase by selective deuteration and selective protonation

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Adenylate kinase from two types of *Escherichia coli* strains, a wild-type strain and a leucine-auxotrophic strain, was purified. On the one hand, growing the leucine-auxotrophic bacteria on a medium containing deuterated leucine yielded *E. coli* adenylate kinase with all leucine residues deuterated. On the other hand, by growing the wild-type bacteria on deuterated medium with phenylalanine, threonine and isoleucine present as protonated specimens, 80% randomly deuterated enzyme with protonated phenylalanine, threonine and isoleucine residues could be prepared. Use of these proteins enabled identification of the spin systems of these amino acid residues in the n.m.r. spectra of the protein.

INTRODUCTION

Adenylate kinase (AK, ATP:AMP phosphotransferase, EC 2.7.3.4) from *Escherichia coli* with a molecular mass of 23.5 kDa catalyses the transfer of the terminal phosphate group of ATP to AMP in the presence of a bivalent metal ion, physiologically Mg^{2+} (Noda, 1973).

The X-ray structure of this protein with bound P^1P^5-bis-(5′-adenosyl) pentaphosphate (AP_5A), a proposed bisubstrate analogue (Lienhard & Secemski, 1973), is known (Müller & Schulz, 1988). Recently it was shown that it is possible to locate at least one of the nucleotide-binding sites of this enzyme in solution by two-dimensional n.m.r. techniques (Vetter *et al.*, 1990). Characterization of nucleotide-binding sites of adenylate kinase by n.m.r. methods and, in the long run, determination of the solution structure of this protein depend largely on the ability to assign the proton resonances sequence specifically to amino acid residues. As a first step, we previously were able to identify most of the spin systems in the aromatic proton spectral region and suggest sequence-specific assignments in this region (Bock *et al.*, 1988).

The assignment of resonances in proteins of molecular mass over 15 kDa is extremely complicated, in particular in the aliphatic region of the spectrum. This is due, in part, to the severe overlap of resonances. Fortunately, biochemical methods can be applied for extraction of information from these protein spectra in addition to the widely used two-dimensional n.m.r. techniques (Ernst *et al.*, 1987; Wüthrich, 1986). Thus deuteration of amino acid residues with aliphatic side chains in an otherwise protonated protein has previously been shown to be a valuable tool in the spin-system identification procedure with dihydrofolate reductase (Searle *et al.*, 1986). In the present paper we describe a method that led to the identification of alipathic spin systems in the two-dimensional spectra of *E. coli* AK. This was done by using selective deuteration of leucine residues in an otherwise protonated protein and selective protonation of phenylalanine, threonine and isoleucine residues in an otherwise deuterated protein.

MATERIALS AND METHODS

Biochemical procedures

Auxotrophic and wild-type *E. coli* K12 bacteria strains were used to incorporate amino acid selectively into the enzyme. For the implementation of selectively deuterated leucine the strain CK600 (*leu⁻ thr⁻ thi⁻*) was used. Deuterated L-leucine was prepared by platinum-catalysed exchange in ^2H_2O. The catalyst Pt, which was obtained by pre-reduction of PtO_2 with $^2H_2$ gas in ^2H_2O at room temperature, L-[$^2H_3$]leucine (pre-exchanged $-N^2H_3^+$ group) and ^2H_2O were sealed under exclusion of O_2 in a glass vessel. The mixture was heated to 100 °C and shaken for 10 days. ^{1}H-n.m.r. spectroscopy (500 MHz, ^2H_2O and equiv. ^{2}HCl) showed that the deuterated leucine obtained was better than 95% exchanged on the β-, γ- and methyl hydrogen atoms. The α-hydrogen was approx. 20% deuterated, which resulted in approx. 20% racemization of the deuterated L-leucine.

The pEMBL plasmid in which the *adk* gene of *E. coli* was cloned (Reinstein *et al.*, 1988) was used in order to overexpress the protein. The transformed cells were grown in special medium (Table 1), containing 100 mg of carbenicillin/l.

The strain JM101a was used for selective protonation. After transformation the bacteria were grown in L-broth containing ampicillin (50 mg/l). It was then transferred to minimal medium [KH_2PO_4 (3 g/l)/Na_2HPO_4 (6 g/l)/NaCl (0.5 g/l)/$MgSO_4$ (0.4 g/l)/ampicillin (50 mg/l), pH 7] containing 2 g of casamino acids (Difco)/l and then to minimal medium without casamino acids but containing succinic acid (5 g/l) as sole carbon source. These bacteria were then progressively adapted to growth in ^2H_2O and use of deuterated succinic acid (Stella, 1973) as carbon source. Transfers from one medium to another had to be achieved at constant temperature (37 °C) and with bacterial growth being in the early exponential phase.

Abbreviations used: *adk*, adenylate kinase gene; AK, adenylate kinase; AP_5A, P_1P^5-bis-(5′-adenosyl) pentaphosphate; COSY, correlated spectroscopy; COSY-θ, COSY with θ reading pulse; DQF-COSY, double-quantum filtered COSY; RCT, relayed coherence transfer spectroscopy.

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Table 1. Ingredients used for growth of bacteria with deuterated leucine for 1 litre of medium

Solution	Composition
Solution A	Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 0.5 g; NH_4Cl, 1 g; pH 7.4
Solution B	2 M-$MgSO_4$, 1 ml ; 40% (w/v) glucose, 10 ml; 1 M-$CaCl_2$, 0.1 ml; 0.01% $FeSO_4$, 1 ml; 0.01% vitamin B_1, 1 ml
Solution C	8% (w/v) sodium pyruvate, 10 ml
Solution D	Amino acids (except leucine) corresponding to their occurrence in *E. coli* (Lehninger, 1983), 5 g; deuterated leucine, 100 mg

Preparative cultures of 18 litres were carried out in a Chemapec 20-litre fermenter at 37 °C in medium containing 80% (v/v) ^2H_2O and succinic acid deuterated to an extent of approx. 93%. The culture containing 2 mg each of phenylalanine, threonine and isoleucine/l was harvested at an A_{600} value of 1.8 and yielded 66 g of bacterial paste.

AK was purified and its activity checked essentially as described previously (Berghäuser & Schirmer, 1978; Barzu & Michelson, 1983; Haase *et al.*, 1988) from the overproducing strains (Brune *et al.*, 1985; Reinstein *et al.*, 1988). The proteins were stored as freeze-dried powders at 253 K after the preparation and after extensive dialysis against 5 mM-sodium phosphate buffer, pH 6.3.

Preparation of n.m.r. samples

For the n.m.r. experiments residual ^1H_2O was exchanged with ^2H_2O by addition of ^2H_2O to a preweighed amount of freeze-dried protein, followed by overnight storage at room temperature in order to exchange labile amide protons. This was followed by freeze-drying and subsequent solution in 99.96% ^2H_2O ('100% D_2O' from Sigma Chemical Co.) to the desired concentration. In the final samples the phosphate buffer concentration was approx. 50 mM at pH 6.3 and the protein concentration was 2.5 mM. Accurate protein concentration was determined before the n.m.r. experiment by using the method of Ehresmann *et al.* (1973).

N.m.r experiments were performed on a Bruker AM 500 spectrometer working at a proton resonance frequency of 500 MHz. Standard procedures were used throughout. The samples were in 5 mm tubes (from Norell), and internal 2,2-dimethyl-2-silapentane-1-sulphonate was used as a reference. The samples were kept at 300 K with a precooled stream of dry air temperature-regulated with a standard Bruker VT 1000 unit. The residual solvent peak was suppressed by permanent (except acquisition) selective irradiation. Two-dimensional experiments, i.e. correlated spectroscopy with a θ reading pulse (COSY-θ; Bax & Freeman, 1981), double-quantum filtered COSY (DQF-COSY; Marion & Wüthrich, 1983; Rance *et al.*, 1983) and relayed coherence transfer spectroscopy (RCT; Eich *et al.*, 1982), were performed according to well-established procedures (Wüthrich, 1986). All two-dimensional spectra were obtained in the phase-sensitive mode with quadrature detection in both dimensions by using the time proportional phase incrementation technique (Marion & Wüthrich, 1983). The essential experimental parameters were identical with those used in previous work (Bock *et al.*, 1988).

RESULTS AND DISCUSSION

Strategy

As a starting point for the analysis of the aliphatic part of the spectrum it is convenient to concentrate on two subregions, namely the high-field region of the valine, leucine and isoleucine side-chain resonances (region A) and the region of the threonine, alanine and glycine side-chain resonances (region B). In the first experiment, where we used leucine-auxotrophic bacteria, the growth medium contained deuterated leucine and was protonated otherwise. Thus it is expected that in the COSY spectrum the resonances of the deuterated leucine residues will no longer be visible and can therefore be distinguished from the proton resonances of the valine residues in the completely protonated enzyme.

In the second experiment, the protein was grown in deuterated growth medium containing protonated phenylalanine, threonine and isoleucine. Accordingly, the n.m.r. spectrum is expected to show resonances of high intensity only for residues of these three amino acids.

Comparison of the two-dimensional experiments with completely protonated AK (wild-type reference spectrum) is then

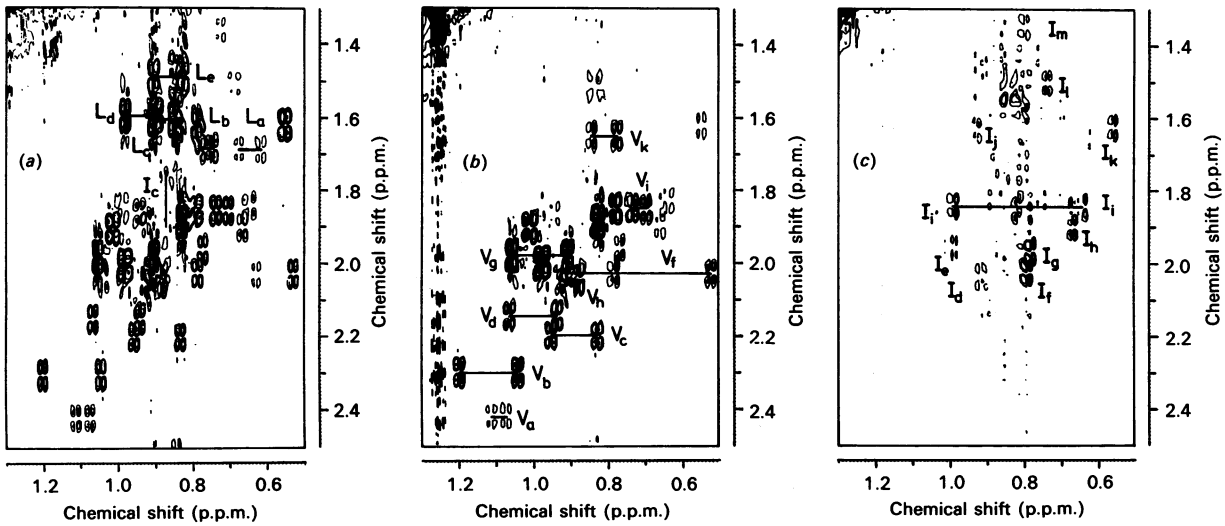

Fig. 1. Spectral analysis of region A

(*a*) COSY-60° of wild-type AK: leucine $C_{(\gamma)}H$–$C_{(\delta)}H_3$ and $C_{(\gamma)}H$–$C_{(\delta')}H_3$ cross-peaks and isoleucine $C_{(\gamma)}H$–$C_{(\delta)}H_3$ and $C_{(\gamma')}H$–$C_{(\delta)}H_3$ cross-peaks are indicated. (*b*) COSY-60° of leucine-deuterated AK: valine $C_{(\beta)}H$–$C_{(\gamma)}H_3$ and $C_{(\beta)}H$–$C_{(\gamma')}H_3$ cross-peaks are indicated. (*c*) COSY-60° of selectively protonated AK: only isoleucine resonances remain.

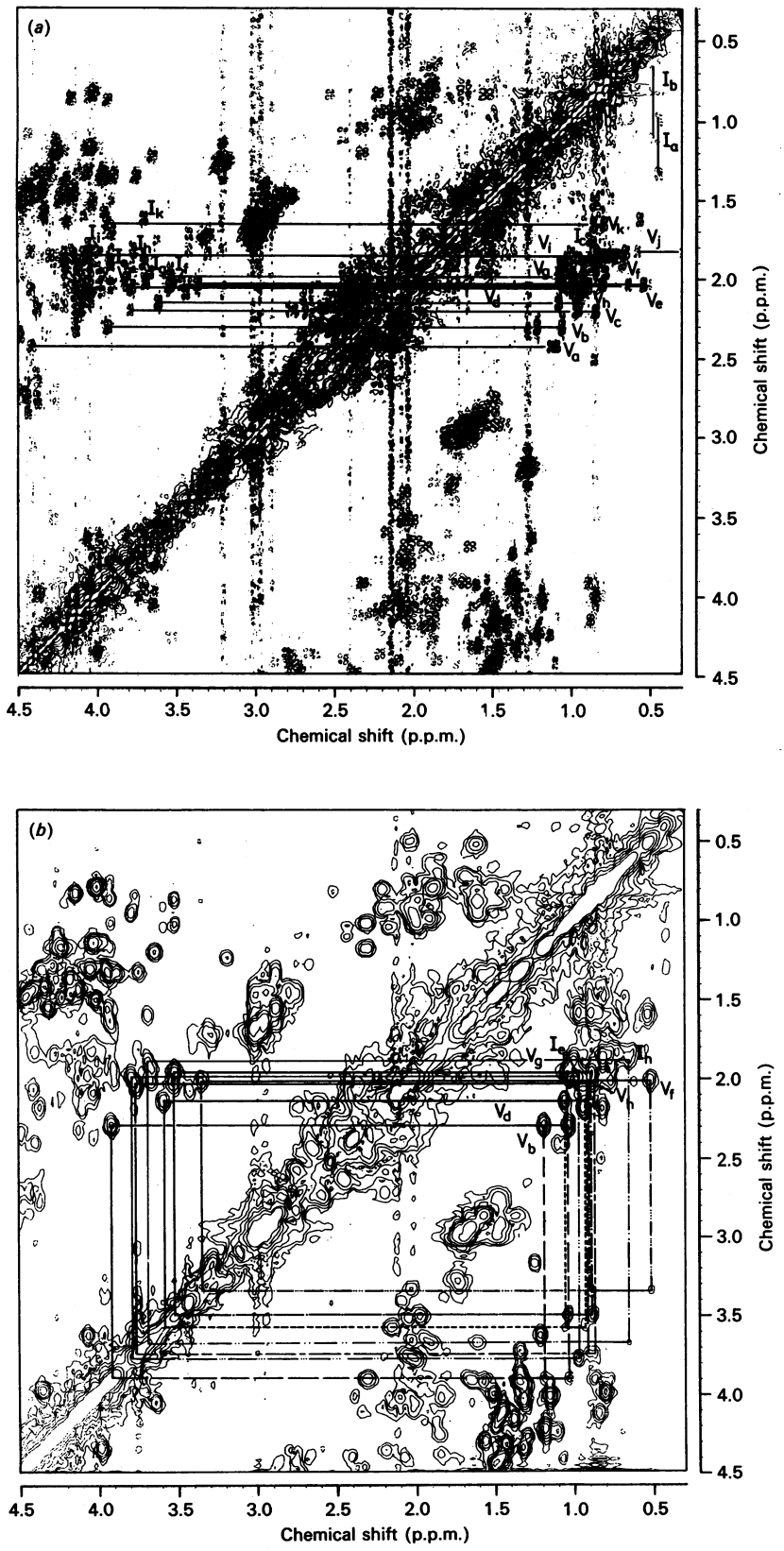

Fig. 2. Aliphatic region of leucine-deuterated AK spectra

(*a*) COSY-60° of the entire aliphatic region of leucine-deuterated AK: connections from the valine $C_{(\alpha)}H$–$C_{(\beta)}H$ cross-peak to the $C_{(\beta)}H$–$C_{(\gamma)}H_3$ cross-peak and to the $C_{(\beta)}H$–$C_{(\gamma')}H_3$ cross-peak as well as from the isoleucine $C_{(\gamma)}H$–$C_{(\delta)}H_3$ cross-peak to the $C_{(\gamma')}H$–$C_{(\delta)}H_3$ cross-peak are indicated. (*b*) RCT spectrum: the continuous lines connect the $C_{(\alpha)}H$–$C_{(\beta)}H$ cross-peaks with the $C_{(\beta)}H$–$C_{(\gamma)}H_3$ and the $C_{(\beta)}H$–$C_{(\gamma')}H_3$ cross-peaks, and the broken lines show $C_{(\alpha)}H$–$C_{(\gamma)}H_3$ and $C_{(\alpha)}H$–$C_{(\gamma')}H_3$ RCT cross-peaks for V_b, V_d, V_f, V_g and V_h and $C_{(\alpha)}H$–$C_{(\gamma)}H_3$ RCT cross-peaks for I_e and $\bar{I}_h$.

expected to yield the unambiguous identification of valine, leucine and isoleucine proton resonances in the high-field aliphatic spectral region: leucine resonances are eliminated by deuteration and isoleucine resonances can be recognized by the selective protonation experiments, so that the assignment of the remaining unidentified resonances in the high-field region of the reference spectrum to valine residues (1.3 p.p.m. to 0.5 p.p.m. in ω_2, 2.5 p.p.m. to 1.3 p.p.m. in ω_1) is possible by exclusion.

In the spectral region of the threonine, alanine and glycine proton resonances we had to rely on a different method. Selective protonation of the enzyme provides the basis for the identification of the threonine resonances. The remaining signals in the region between 1.7 p.p.m. and 0.7 p.p.m. in ω_1 and between 4.8 p.p.m. and 3.5 p.p.m. in ω_2 can then be identified as alanine spin systems. The remaining resonances in the region between 4.8 p.p.m. and 3.5 p.p.m. in ω_1 and in ω_2 are the glycine proton signals.

Spectral analysis

Region A. In Figs. 1 and 2 the aliphatic region that contains the resonances of the branched amino acids valine, leucine and isoleucine is shown. Comparison of parts of the spectrum of the selectively deuterated protein (Figs. 1*b* and 2*a*) and the reference spectrum of the protein grown in protonated medium (Fig. 1*a*) leads to identification of 11 valine, five leucine and three isoleucine spin systems (Tables 2–4). For the valine spin systems labelled V_b, V_d, V_f, V_g and V_h the RCT cross-peaks can be found as well (Fig. 2*b*). Comparison of Fig. 1(*c*) with Figs. 1(*a*) and 1(*b*) reveals ten isoleucine $C_{(\beta)}H$–$C_{(\gamma)}H_3$ and one $C_{(\beta)}H$–$C_{(\gamma)}H$ cross-peaks. Two of these (I_e and I_h) also show $C_{(\alpha)}H$–$C_{(\gamma)}H_3$ RCT cross-peaks (Fig. 2*b*). For six isoleucine spin systems the $C_{(\alpha)}H$–$C_{(\beta)}H$ cross-peaks were also found (Fig. 3*b*). Neither of the pairs of $C_{(\gamma)}H_2$–$C_{(\delta)}H_3$ cross-peaks (I_{a-c}) can be related definitely to a $C_{(\beta)}H$–$C_{(\gamma)}H_3$ cross-peak (I_{d-m}) since the RCT spectrum provided only very few additional connectivities, a fact that may well be due to the high molecular mass of the protein.

Region B. Comparison of Fig. 3(*a*) (wild-type AK) with Fig. 3(*b*) (selectively protonated AK) reveals 12 threonine, 17 alanine and five glycine residues (Table 5 and 6). Although the sequencing

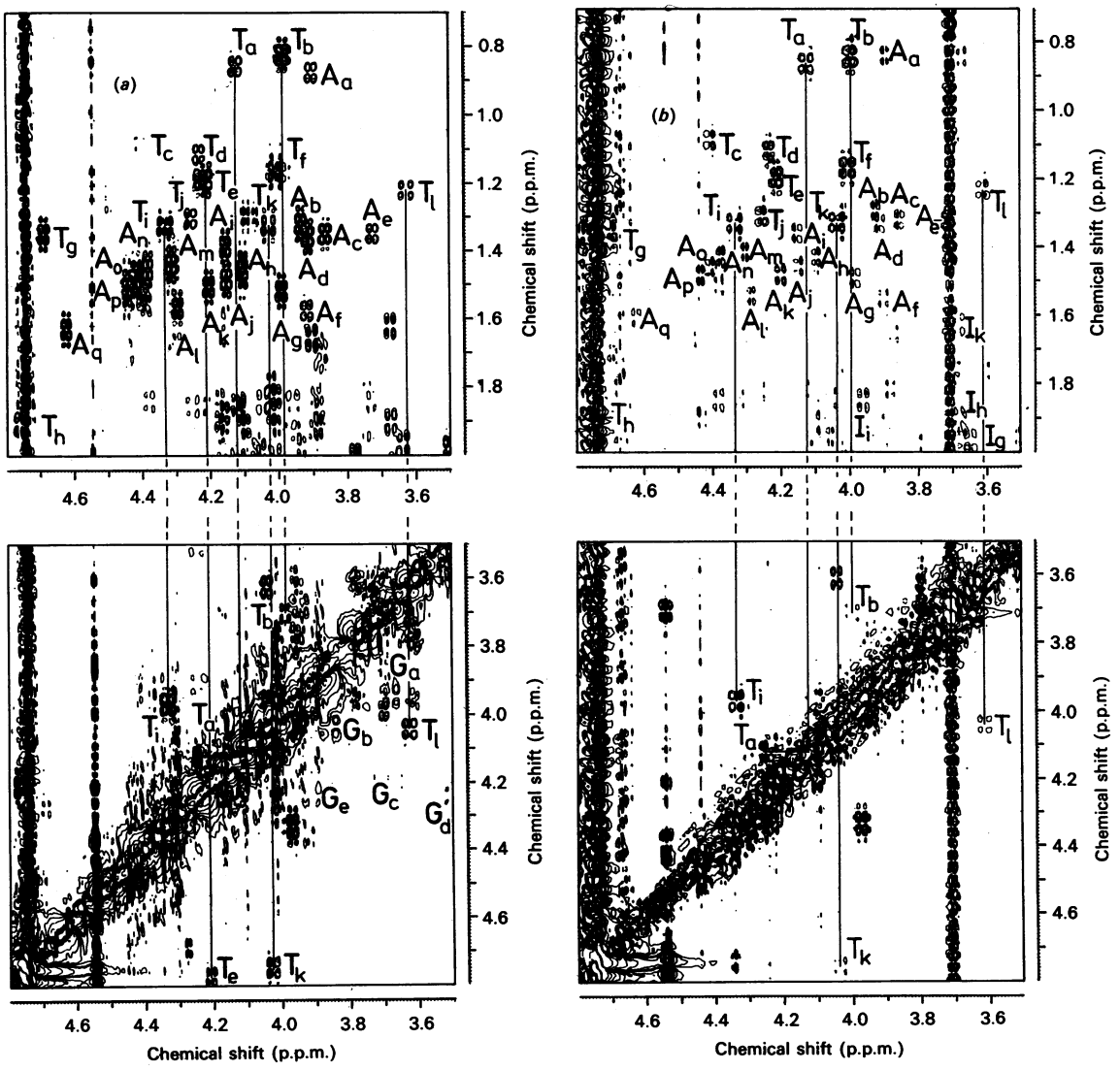

Fig. 3. Spectral analysis of region B

(*a*) COSY-60° spectrum of wild-type AK. Indicated are (upper spectrum) threonine $C_{(\beta)}H$–$C_{(\gamma)}H_3$ and alanine $C_{(\alpha)}H$–$C_{(\beta)}H_3$ cross-peaks and (lower spectrum) glycine $C_{(\alpha)}H$–$C_{(\alpha')}H$ and threonine $C_{(\alpha)}H$–$C_{(\beta)}H$ cross-peaks. (*b*) COSY-60° spectrum of wild-type AK from bacteria grown in deuterated medium in the presence of protonated phenylalanine, threonine and isoleucine. Indicated are (upper spectrum) threonine $C_{(\beta)}H$–$C_{(\gamma)}H_3$ and alanine $C_{(\alpha)}H$–$C_{(\beta)}H_3$ cross-peaks and (lower spectrum) threonine $C_{(\alpha)}H$–$C_{(\beta)}H$ cross-peaks

Table 2. Chemical shifts of valine residue resonances

Valine residue	Chemical shift (p.p.m.)			
	$C_{(\alpha)}H$	$C_{(\beta)}H$	$C_{(\gamma)}H$	$C_{(\gamma')}H$
V_a	4.37	2.42	1.11	1.07
V_b	3.90	2.30	1.20	1.04
V_c	3.75	2.20	0.95	0.83
V_d	3.58	2.15	1.06	0.94
V_e	3.42	2.03	0.63	0.51
V_f	3.34	2.02	0.90	0.53
V_g	3.51	1.98	1.05	0.90
V_h	3.75	2.04	0.93	0.88
V_i	4.21	1.85	0.73	0.70
V_j	–	1.82	0.57	0.30
V_k	3.90	1.65	0.85	0.78

Table 3. Chemical shifts of leucine residue resonances

Leucine residue	Chemical shift (p.p.m.)		
	$C_{(\gamma)}H$	$C_{(\delta)}H$	$C_{(\delta')}H$
L_a	1.69	0.68	0.62
L_b	1.68	0.75	0.76
L_c	1.61	0.90	0.85
L_d	1.60	0.98	0.89
L_e	1.49	0.91	0.83

Table 4. Chemical shifts of isoleucine residue resonances

Isoleucine residue	Chemical shift (p.p.m.)					
	$C_{(\alpha)}H$	$C_{(\beta)}H$	$C_{(\gamma)}H_3$	$C_{(\gamma)}H$	$C_{(\gamma)}H$	$C_{(\delta)}H_3$
I_a	–	–	–	1.34	1.00	0.44
I_b	–	–	–	1.12	0.84	0.45
I_c	–	–	–	1.89	1.75	0.87
I_d	–	2.03	0.92	–	–	–
I_e	3.51	1.95	0.99	–	–	–
I_f	3.49	2.02	0.79	–	–	–
I_g	3.64	1.96	0.77	–	–	–
I_h	3.67	1.90	0.67	–	–	–
I_i	3.96	1.85	0.65	0.99	–	–
I_j	–	1.63	0.93	–	–	–
I_k	3.67	1.62	0.56	–	–	–
I_l	–	1.51	0.74	–	–	–
I_m	–	1.35	0.80	–	–	–

Table 5. Chemical shifts of threonine residue resonances

Threonine residue	Chemical shift (p.p.m.)		
	$C_{(\alpha)}H$	$C_{(\beta)}H$	$C_{(\gamma)}H$
T_a	4.25	4.12	0.86
T_b	3.70	3.98	0.82
T_c	–	4.40	1.08
T_d	–	4.23	1.12
T_e	4.76	4.21	1.19
T_f	–	4.00	1.17
T_g	–	4.69	1.35
T_h	–	4.76	1.90
T_i	3.96	4.33	1.33
T_j	–	4.26	1.30
T_k	4.74	4.03	1.33
T_l	4.04	3.63	1.22

Table 6. Chemical shifts of alanine and glycine residue resonances

Alanine residue	Chemical shift (p.p.m.)		Glycine residue	Chemical shift (p.p.m.)	
	$C_{(\alpha)}H$	$C_{(\beta)}H$		$C_{(\alpha)}H$	$C_{(\alpha')}H$
A_a	3.90	0.88	G_a	3.68	3.94
A_b	3.93	1.32	G_b	3.86	4.03
A_c	3.86	1.35	G_c	3.67	4.21
A_d	3.91	1.36	G_d	3.50	4.23
A_e	3.73	1.35	G_e	3.91	4.24
A_f	3.92	1.58			
A_g	3.99	1.52			
A_h	4.10	1.46			
A_i	4.16	1.39			
A_j	4.15	1.48			
A_k	4.20	1.51			
A_l	4.29	1.57			
A_m	4.31	1.44			
A_n	4.38	1.46			
A_o	4.41	1.49			
A_p	4.44	1.51			
A_q	4.62	1.63			

of the DNA coding for the *E. coli* AK showed that this enzyme contained only 11 threonine residues, the region symmetric to the one shown in the upper part of Fig. 3(*a*) showed 12 very intense resonances as compared with the very-low-intensity resonances of the alanine protons. Accordingly, it is no easy matter to decide which of the spin-system identifications was erroneous. In addition, the $C_{(\alpha)}H$–$C_{(\beta)}H$ cross-peaks can be detected for the threonine spin systems labelled T_a, T_b, T_e, T_i, T_k and T_l. The β-proton resonance was found at lower field than the $C_{(\alpha)}$–proton resonance for T_b and T_l. Threonine $C_{(\alpha)}H$–$C_{(\beta)}H$ cross-peak fine structures are very characteristic: depending on whether the $C_\alpha H$ peak is located low field of the $C_{(\beta)}H$ peak or not, the cross-peak fine structure shows only the active coupling $J_{\alpha\beta}$ along ω_2 and the passive couplings $J_{\beta\gamma}$ along ω_1, or vice versa, a fact that may be derived easily from spectrum simulations (Bock & Rösch, 1987). This leads to clearly distinguishable cross-peak shapes in spite of the fact that the cross-peak fine structure cannot be resolved clearly in all cases.

Because of the passive couplings, the threonine $C_{(\alpha)}H$–$C_{(\beta)}H$ cross-peak covers a rectangular area and changes its appearance when we consider the symmetric cross-peak at the position where ω_1 and ω_2 are interchanged (Bock-Möbius, 1989). Distinction of a threonine $C_{(\alpha)}H$–$C_{(\beta)}H$ cross-peak from a glycine cross-peak, which has only one active coupling and therefore covers a square area on both sides of the diagonal, is always possible when both of the symmetric cross-peaks can be found.

Aromatic region. The same resoning was used to distinguish tyrosine from phenylalanine spin systems in the aromatic region: the spectrum of the selectively protonated protein show only phenylalanine proton signals in the aromatic spectral region. Although for this region no additional information was obtained, the identification of tyrosine and phenylalanine residues as discussed in Bock *et al.* (1988) was confirmed.

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