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Structure-function relationship in *Escherichia coli* translational initiation factors

Characterization of IF1 by high-resolution ^{1}H-NMR spectroscopy

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Escherichia coli translational initiation factor IF1 was studied by ^{1}H-NMR spectroscopy at 400 MHz. IF1 displays a very well resolved spectrum in both aromatic and aliphatic regions. Other spectral characteristics include relatively narrow resonance lines and lack of relevant cross-relaxation phenomena. The resonances of the aromatic residues, in particular of the two His and two Tyr, were assigned by selective chemical modifications and spectroscopic techniques to individual residues in the protein sequence. The relative mobility of various residues of IF1 has been evaluated on the basis of the spin-lattice relaxation times which are rather short and homogeneous. Overall the factor appears to have a complex secondary and tertiary structure and to be a flexible protein whose residues have a high degree of internal mobility.

Translation; Initiation factor 1; ^{1}H-NMR; Relaxation time; Protein structure

1. INTRODUCTION

Initiation factor IF1 (M_r 8119), the smallest of the three protein factors required for translational initiation in *Escherichia coli* (reviews [1,2]), consists of 71 amino acid redidues of known sequence [3]. A gene coding for a protein with similar size and homologous primary structure has recently been found in the chloroplast DNA of two distant plants [4,5]. During protein synthesis, IF1 binds, presumably in combination with the other two initiation factors, to the free 30 S ribosomal subunits [6]. Omission of IF1 from reaction mixtures containing all purified components required for protein synthesis results in a much reduced translational rate [7]. This effect can be explained by the kinetic role of IF1 in accelerating the rate of formation of the 30 S initiation complex [8,9]. Upon the joining of a 50 S ribosomal subunit to the 30 S initiation complex, which generates the 70 S initiation complex, IF1 is ejected from the 30 S particle [6].

A better understanding of the mode of action of IF1 awaits knowledge of its structure and active sites. A step towards this end is the characterization of IF1 by high-resolution NMR spectroscopy and the assignment of its aromatic spectrum which are presented in this paper.

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2. MATERIALS AND METHODS

Purified, electrophoretically homogeneous IF1 was prepared from *E. coli* MRE600 cells according to [10].

NMR spectra were run with a Bruker NMR WH-400 spectrometer using a 30° pulsewidth. Free induction decays (FIDs) were collected on 16 K memory for 1000–2000 scans and then Fourier transformed. Unless otherwise specified, no filtering was used. The dependence of chemical shift on pH was measured following addition of minute amounts of DCl or NaOD to samples. The pH was measured in the NMR tubes with a microelectrode and meter readings were used without further correction.

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Double-resonance experiments were performed using standard homo-gated decoupling techniques. Spin-lattice relaxation times were measured by the inversion recovery T-180-τ-90-FID pulse sequence; T (waiting time between sequences) was chosen to be sufficiently long to allow the spin system to relax completely after the acquisition. To observe the relaxation behavior and to measure the related time constants of the individual resonances (T_1), the τ value ranged from about 1 s to a few tenths of a millisecond.

3. RESULTS AND DISCUSSION

3.1. *The 400 MHz ^{1}H-NMR spectrum of IF1*

The spectrum of IF1 is very well resolved in both aromatic and aliphatic regions (fig.1). As shown below, most of the aromatic resonances could be assigned by spectroscopic and protein chemical methods to specific amino acid residues. In the aliphatic region, however, due to the complexity of the spectrum which contains several bands, single resonances and a discrete number of ring-current shifted methyl (RCSM) resonances, only a few tentative assignments [11,12] could be made.

3.2. *pH dependence of the ^{1}H-NMR spectrum*

The pH range amenable for investigation of IF1 is pH 5.5–8.5; above or below these values and at the protein concentrations necessary for NMR spectroscopy, precipitation occurs. Within this pH range, the C-2 and C-4 protons of the two histidines of the molecule undergo substantial changes in chemical shift, unaccompanied by changes in other regions of the spectrum.

The titration of the two C-2 protons is presented in fig.2A,B. The $\log(\delta_a - \delta_{obs})/(\delta_{obs} - \delta_b)$ vs pH plots are linear for both His residues, indicating that, at least within the pH range investigated, there are no co-titrating groups. The measured pK values were 6.68 ± 0.05 for His A (fig.2A) and 6.35 ± 0.05 for His B (fig.2B). Unlike the case with the C-2 protons, the chemical shift changes of the C-4 proton resonances cannot be followed throughout the pH range due to partial or complete overlapping with other resonances.

3.3. *Assignment of the His resonances*

IF1 contains two His residues at positions 29 and 34 of its primary structure [3]. Owing to the difference in their pK values (cf. fig.2A,B), assignment of their resonances in the spectrum was obtained following dye-sensitized photooxidation at different pH values. Since photooxidation takes place on the deprotonated imidazole ring and since

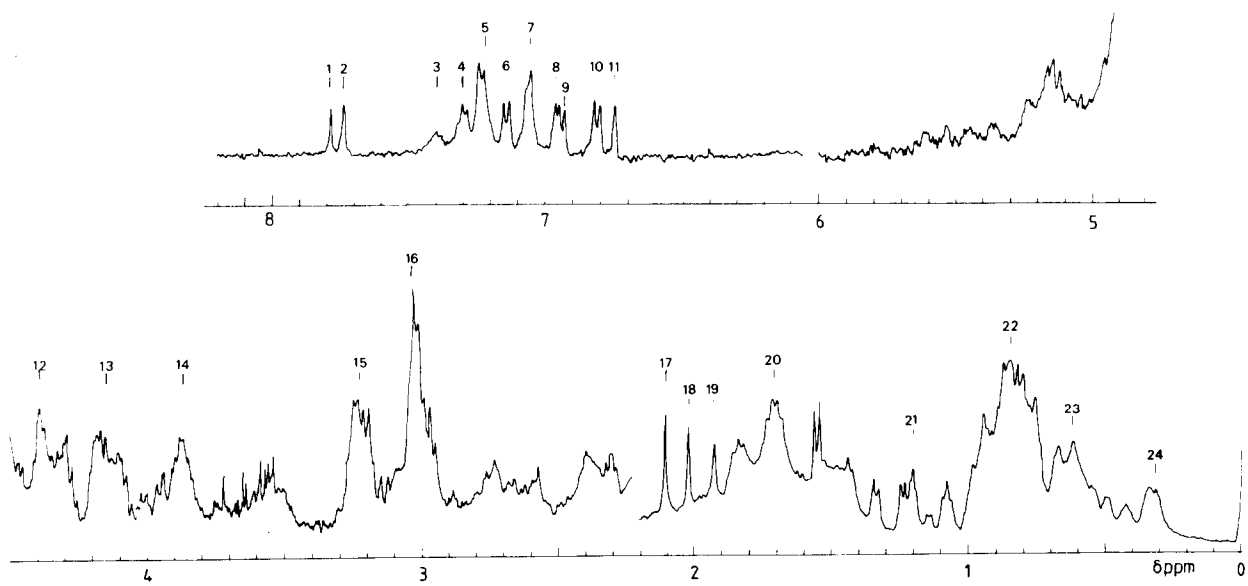

Fig.1. ^{1}H-NMR spectrum of IF1. The spectrum of IF1 at 400 MHz at a concentration of 5×10^{-4} M was obtained as described in section 2. The 2.3–0 ppm region was reduced in intensity by a factor of 2. Resonances are labelled with numbers corresponding to the tentative assignments of table 1.

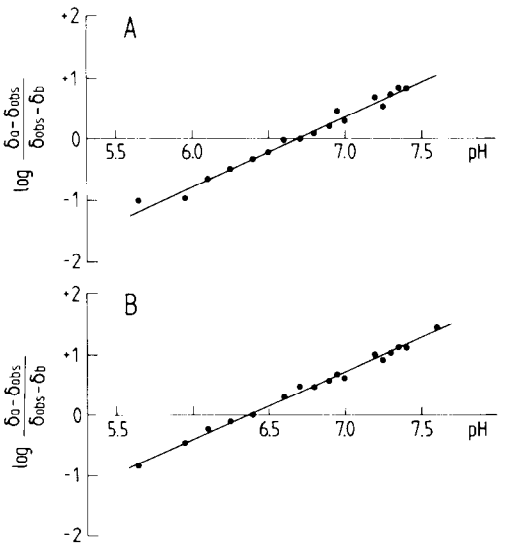

Fig.2. Titration of the histidine residues. Dependence on pH of the chemical shift of the C-2 resonances of His A and B of IF1 is reported as a plot of $\log(\delta_a - \delta_{obs})/(\delta_{obs} - \delta_b)$ vs pH (b, basic; a, acid) to obtain the pK values of the two residues.

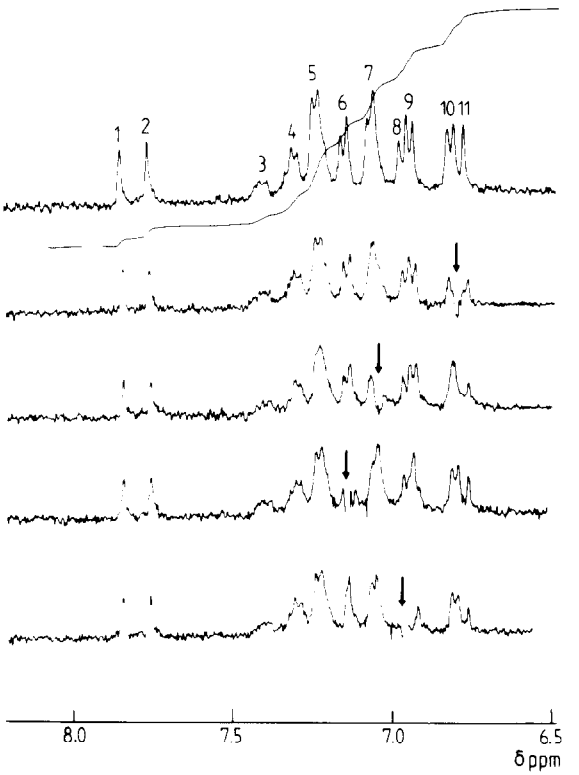

Fig.3. Assignment of the spin sysem of Tyr resonances. The assignment of the spin system of the Tyr resonances of IF1 was performed by homonuclear decoupling. Arrows indicate the selective irradiation.

we found that His 34 becomes photooxidized at lower pH than His 29 [13], we conclude that His 34 has a lower pK than His 29 and therefore its C-2 resonance corresponds to that denoted His B in the spectrum. Consequently, the C-2 resonance of His 29 must correspond to that denoted His A. The assignment of the C-4 protons of these residues was made possible by the inversion recovery experiment presented below.

The values of the spin-lattice relaxation times obtained by the inversion recovery pulse sequence (see below) indicate that the two His residues or at least their aromatic rings are engaged to different extents in the tertiary structure of the molecule. In fact, the resonances due to the protons of the imidazole ring of His 34 show relaxation times shorter than those measured for the ring protons of His 29, indicating that the latter residue has a higher degree of mobility than His 34. The lower pK value found for the imidazole protons of His 34 also suggests that this residue is most likely involved in hydrogen bonding within the tertiary structure of the protein.

3.4. *Assignment of the Tyr resonances*

The spin systems of the 2,6 and 3,5 protons of the two Tyr residues have been identified by homonuclear decoupling (fig.3). The assignment of these residues within the primary structure of the protein was obtained by selective chemical modification experiments.

The method used for identification of the Tyr resonances (i.e. comparison of the spectra recorded before and after nitration of tyrosines with tetranitromethane) has been successfully applied in other cases [14].

The spectrum of the modified protein (fig.4b) shows 'new' resonances (stippled areas) which partly overlap those of the native protein (fig.4a). The new resonances are due to the protons of the nitro-substituted aromatic rings. In fact, nitration causes the disruption of the symmetry of the AA'BB' spin system of the Tyr ring with the establishment of two ABC spin systems depending on which carbon is nitrated. The precise assignment of the resonances arising from the nitration of the protein is beyond the scope of this work.

However, since the two Tyr residues of IF1 (position 43,59) react at different rates [15], we were able to assign the individual resonances to specific Tyr residues by comparing the spectra recorded after different times of reaction. For this purpose, we performed the deconvolution with a Lorentzian line shape of the aromatic resonances. An example of a computer-generated spectrum obtained as a sum of the deconvoluted resonances is presented in fig.4c. The decrease in integrated intensities of the 3,5 protons of the two Tyr residues as a function of reaction time was followed upon normalization of the resonances (fig.5). As seen from the figure, the resonance intensities of the two Tyr residues decrease at different rates, the one labelled 'A' decreasing faster than 'B'. Since it has been found that Tyr 59 is more accessible to chemical modification than Tyr 43 [15], we conclude that Tyr A and Tyr B correspond to Tyr 59 and Tyr 43, respectively.

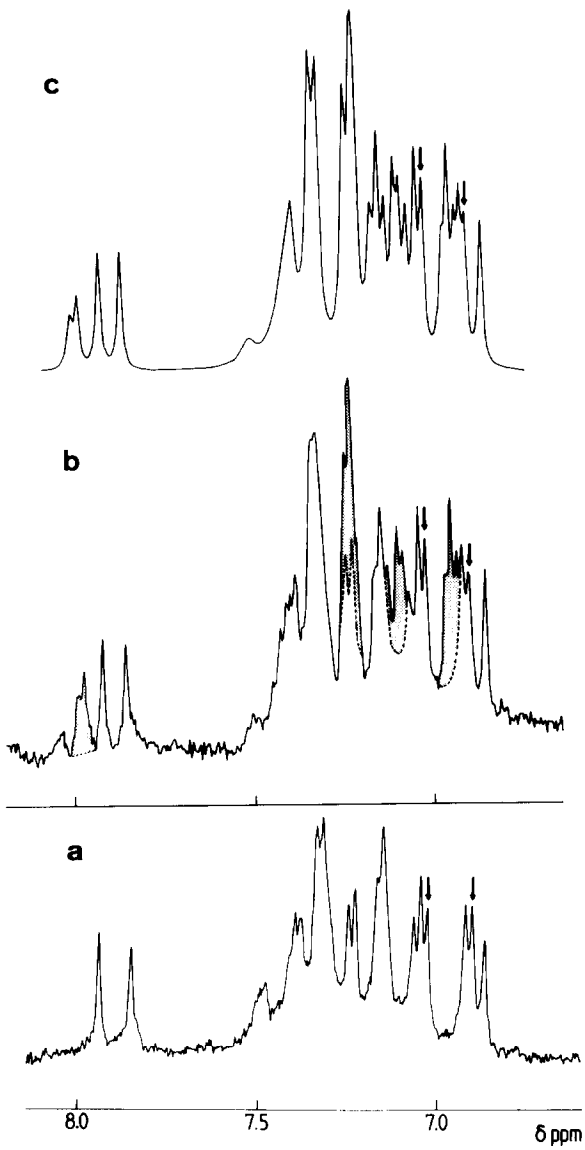

Fig.4. Nitration of the Tyr residues of IF1. (A) Spectrum of the native protein. (B) Spectral changes induced by nitration (10 min) of the Tyr residues are presented (stippled area). (C) Fitting of the experimental spectrum with a sum of Lorentzian lineshapes. Arrows indicate resonances used for evaluation of the extent of nitration rate presented in fig.5.

3.5. *Spin-lattice relaxation times*

The spin-lattice relaxation times (T_1) for individual proton resonances and bands were determined by inversion recovery. The stacked plot of the aromatic region of this experiment from which we were also able to assign the C-4 proton resonances of the two His residues is presented in fig.6. The plots of magnetization vs the 14 increasing τ values chosen for this experiment yielded straight lines for all resonances except for those designated 3, 5, 23 and 24. The first two bands, assigned to Phe ring protons, showed a convex deviation from linearity; the other two, attributable to RCSM protons, showed a concave

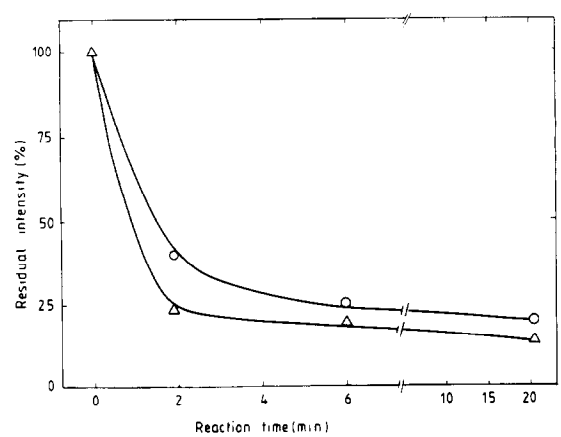

Fig.5. Nitration of the Tyr residues. From the fitting of a computer-generated spectrum like that shown in fig.4C, with the experimental spectra, the relative integrated intensity due to the doublet of the 3,5 protons of the unmodified Tyr residues was evaluated. This value is presented as a function of the reaction time with TNM.

deviation. The overall good linear fitting of the data indicates the absence of extensive cross-relaxation phenomena [16]. The relaxation times (T_1) obtained from the magnitization decay plots are presented in table 1. The values are generally high and very similar for all the amino acid redisues, suggesting that the protein is endowed with a rather high degree of internal mobility. The resonance of His B (His 34) however, displays a much shorter relaxation time than His A (His 29), as is also evident from the spectra shown in fig.6. This behavior indicates that His 34 is characterized by a much lower degree of internal mobility than His 29.

4. CONCLUSIONS

A ^{1}H-NMR spectroscopic study of translational initiation factor IF1 has been presented. Several spectral features such as the abundant ring current shifted methyl resonances and the evidence of clearly distinct chemical and magnetic environments for the two His and Tyr residues of the protein indicate that, in spite of its size, IF1 is endowed with a rather complex secondary and tertiary structure.

The size of this protein, the good resolution achieved and the considerable spreading of its resonances in both aromatic and aliphatic regions of the spectrum as well as the rather narrow lineshapes suggest that the application of 2D NMR spectroscopic techniques to this protein may eventually lead to the elucidation of its 3D structure in solution.

The recent assembly of a synthetic gene encoding IF1 designed to allow facile cassette mutagenesis and the very large amount of IF1 obtainable from an overproducing strain carrying this gene [17] should facilitate this task. Furthermore, site-directed mutagenesis, combined with the 2D NMR technique, may not only represent a method for checking the spectral assignments, but may also offer the opportunity to investigate the

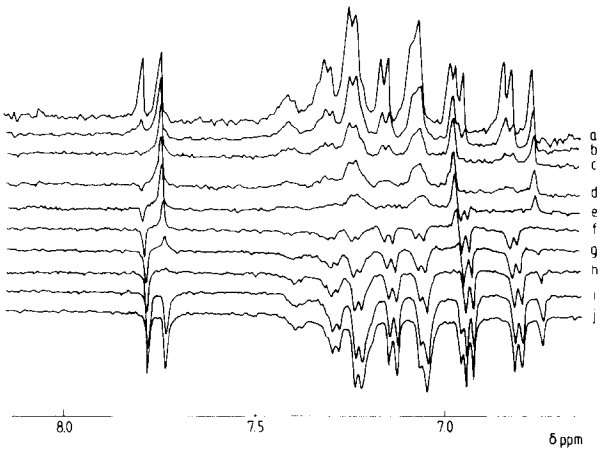

Fig.6. T_1 relaxation times. Stacked plots of the inversion recovery experiment by the 180–τ–90 pulse sequence for measuring the T_1 spin-lattice relaxation times of the aromatic region of the spectrum are shown. τ values: (a) 2.5, (b) 1.2, (c) 0.9, (d) 0.7, (e) 0.55, (f) 0.45, (g) 0.2, (h) 0.1, (i) 0.05, (j) 0.02 s.

Table 1

T_1 spin-lattice relaxation times of IF1 resonances[a]

Resonance no.[b]	Chemical shift (δ, ppm)	Assignment	T_1 (s)[c]
1	7.79	C-2 His 29 (A)	1.45
2	7.73	C-2 His 34 (B)	0.49
3	7.39	∅ Phe	1.32[f]
4	7.28	∅ Phe	1.22
5	7.22	∅ Phe	1.10[f]
6	7.14	C-2,6 Tyr 59 (A)	1.25
7	7.06	C-2,6 Tyr 43 (B)[d]	1.30
8	6.97	C-4 His 34 (B)	0.66
9	6.92	C-3,5 Tyr 59 (A)	1.37
10	6.82	C-3,5 Tyr 43 (B)	1.25
11	6.76	C-4 His 29 (A)	1.12
12	4.39	αCH protons	0.85
13	4.13	αCH Ile, Ala	0.80
14	3.86	αCH Gly	0.50
15	3.23	δCH_2 Arg	0.46
16	3.02	ϵCH_2 Lys	0.48
17	2.12	CH_3-S Met	0.78
18	2.02	CH_3-S Met	0.83
19	1.93	CH_3-S Met	0.82
20	1.71	CH_2 Ala, Arg, Lys	0.52
21	1.20	CH_3 Thr	0.42
22	0.85	CH_3 protons	0.50
23	0.60	CH_3 RCSM[e]	0.47[g]
24	0.32	CH_3 RCSM[e]	0.50[g]

[a] Relaxation times were determined as described in section 2 from a magnetization decay plot obtained by inversion recovery experiment

[b] Numbering is the same as that shown in fig.1

[c] Values are ± 10%

[d] This resonance also contains two protons of a Phe ring

[e] RCSM ring current shifted methyl

[f] Resonance showing convex deviation from linearity in the magnetization decay plot

[g] Resonance showing concave deviation from linearity in the magnetization decay plot

effect of single amino acid replacements on the secondary and tertiary structure. These correlations, in combination with data on the biological effects of these manipulations, can ultimately yield important information concerning the relationships between structure and function of this factor.

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