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High resolution ^{1}H-n.m.r. study of the interaction between initiation factor IF1 and 30S ribosomal subunits

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Interaction between *Escherichia coli* translational initiation factor IF1 (mol. wt. 8119) and 30S ribosomal subunits was followed by high resolution ^{1}H-n.m.r. spectroscopy. Upon gradual addition of increasing yet largely sub-stoichiometric amounts of biologically active deuterated 30S ribosomal subunits, selective line broadenings and chemical shift changes were observed against the background of the gradual disappearance of the whole spectrum. At the highest 30S:IF1 ratio attained (0.25), all the resonance lines were broadened beyond meaningful detection. This behaviour, which can be partly reversed by increasing the ionic strength and/or the temperature, is due to the interaction between IF1 and the 30S ribosomal subunits, and can be explained by the existence of a medium-fast exchange dynamics between free and bound factor. The selective effects observed during titration with 30S ribosomal subunits shed some light on the mode of interaction of IF1 with 30S ribosomal subunits. At least one of the two His residues of the factor appears to be involved in the binding, since it undergoes a low-field change of chemical shift and becomes totally immobilized in the IF1-30S complex. Also strongly implicated in the interaction with 30S are more than one Ser and Arg residue and probably one lysine. Additional effects of the interaction of IF1 with ribosomes are a drastic reduction in the intensity of the ring current upfield shifted methyl resonances and mobilization of a previously rotationally hindered phenylalanine ring.

Key words: initiation factor/n.m.r./nucleic acid-protein interaction/protein synthesis/ribosomes

Introduction

Escherichia coli translational initiation factor IF1 is the smallest of the three initiation factors [mol. wt. from primary structure = 8119, Pon *et al.* (1979)], and the one whose function remains most elusive; in fact, IF1 stimulates the activities of IF2 and IF3, but does not appear to have any specific role of its own. Recently, initial rate kinetic studies of formation of the 30S initiation complex showed that the addition of IF1 to an otherwise complete system increases the V_{max} of the rate-limiting transition in ternary complex formation, but does not affect the affinity of the 30S ribosomal subunit for either mRNA or initiator tRNA (Gualerzi and Pon, 1981; Pon and Gualerzi, in preparation). In addition, fluorescence stopped-flow experiments indicated that IF1 alone has no detectable effect on the rate of ternary complex formation, but it produces a 2- or 4-fold increase when IF3, or IF2-GTP or both are present (Wintermeyer and Gualerzi, 1983).

Equilibrium binding studies by means of airfuge centrifugation using radioactive factor showed that each 30S ribosomal subunit can bind one molecule of IF1 with an association constant ranging from 10^5/M to 10^8/M depending upon the ionic strength and that the binding affinity is enhanced by the presence of IF3 and IF2 (Gualerzi and Pon, 1981; Pawlik *et al.*, in preparation). Interaction of IF1 with the 50S ribosomal subunit was also observed, but the association constant was found to be approximately two orders of magnitude lower than that determined for the 30S particles. Interaction with 70S was not observed; on the contrary, the addition of 50S ribosomal subunits to 30S particles bearing IF1 promoted the release of the factor, thus suggesting that the physiological recycling of IF1 occurs when the 30S and 50S ribosomal subunits join to form a 70S initiation complex (Gualerzi and Pon, 1981; Pawlik *et al.*, in preparation).

IF1 has weak RNA binding capacity (Schleich *et al.*, 1980; Bruhns, 1980) and its ribosomal binding appears to be primarily electrostatic. Although these facts may suggest that the interaction between factor and ribosomes could be due to an interaction with the rRNA, no data nor clear indication exist concerning the molecular nature of this interaction; also very little is known concerning the region or the amino acid residues of the IF1 molecule involved in the interaction with ribosomes.

Here we address these problems by means of high resolution ^{1}H-n.m.r. spectroscopy. This technique is particularly suitable for structural studies on some molecules of biological interest, such as peptides and small proteins and nucleic acids. If the three-dimensional structure of these molecules is known from crystal X-ray diffraction, n.m.r. spectroscopy is essential for establishing its correspondence in solution, in its refinement to the proton level and in studying molecular flexibility and dynamics. Even when X-ray crystallography is not available, n.m.r. spectroscopy can yield important structural information. In the case of IF1, its 400 MHz ^{1}H-n.m.r. spectra appear particularly interesting and well-resolved (for a protein of its size at the accessible concentrations), so that significant structural information concerning this protein has been collected (Paci *et al.*, in preparation). Much more difficult, however, is the study of biological interactions between macromolecules, especially if, as in the present case, the interaction occurs between a protein molecule of moderate size available in limited supply and a cellular organelle, the 30S ribosomal subunit, composed of >20 molecules with a particle weight of nearly 10^6.

For the study of IF1-ribosome interaction by ^{1}H-n.m.r. spectroscopy, we have chosen to use 80 – 90% deuterated ribosomal subunits, which produce no spectrum, so that the only signal seen would be that of the factor. Concerning the method of studying the interaction, two different approaches are, in principle, possible. First, one could try to obtain a stoichiometric amount of the IF1-ribosome complex and compare the spectrum so obtained with that of free IF1. In the second approach, one could titrate the factor with increasing amounts of ribosomes and look for the appearance of any particular spectral change which might be related to a specific interaction between a given amino acid residue and

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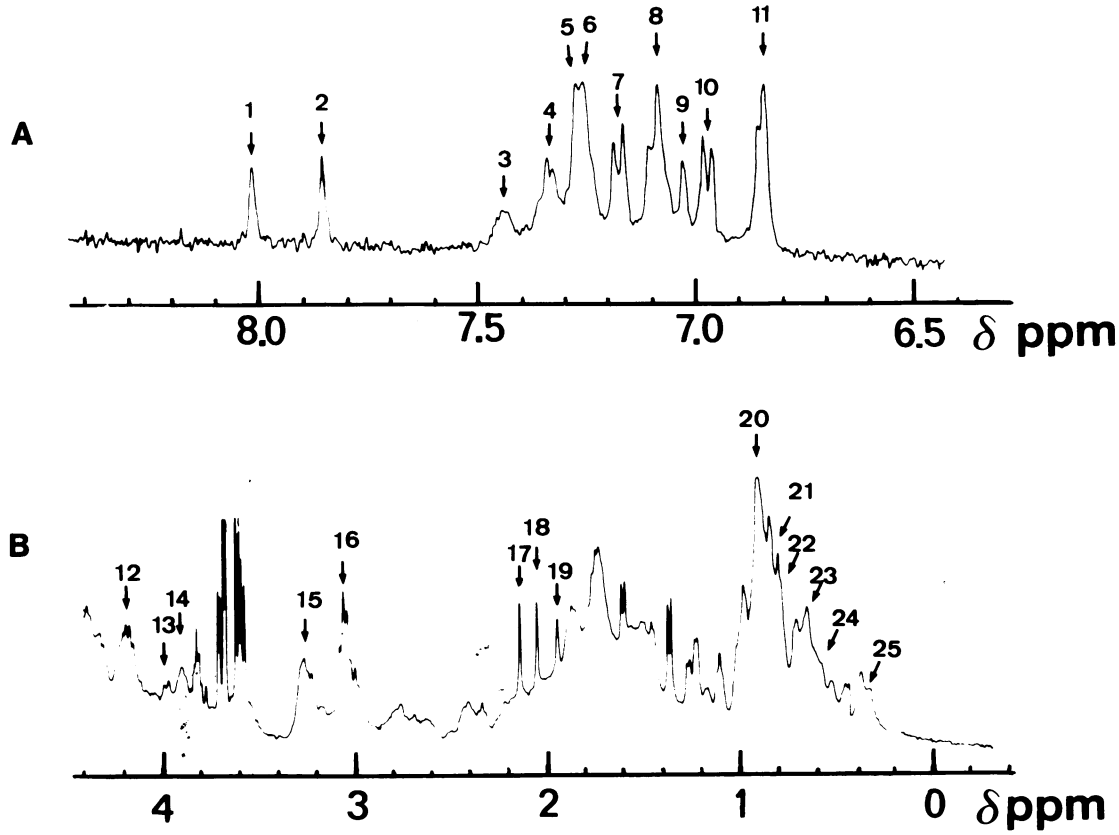

Fig. 1. 400 MHz ^{1}H-n.m.r. spectrum of *E. coli* IF1. Spectral conditions were those described in Materials and methods with an IF1 concentration $\cong 5 \times 10^{-4}$ M. **A**, aromatic protons region; **B**, aliphatic protons region. The numbered arrows indicate the following: **1**, His A C-2 proton; **2**, His B C-2 proton; **3,4,5,6**, Phe A ϕ protons; **4,5,6**, Phe B ϕ protons; **7**, Tyr A 2,6 protons; **8**, Tyr B 2,6 protons and an unassigned resonance; **9**, His A C-4 proton; **10**, Tyr A 3,5 protons; **11**, Tyr B 3,5 protons and His B C-4 proton; **12**, α-CH and β-CH_2; **13,14**, Ser β-CH_2; **15**, Arg δ-CH_2; **16**, Lys ϵ-CH_2; **17,18,19**, Met ϵ-CH_3; **20,21,22**, CH_3 protons; **23,24,25**, upfield ring current shifted CH_3 resonances. δ p.p.m. are referred to 2,2-dimethylsilapentane 5-sulphonic acid.

the ribosomes. Aside from the fact that the first approach is not entirely feasible, due to the concentration barrier imposed by the relatively low 'solubility' of the 30S ribosomes ($\cong 4 \times 10^{-5}$ M), we tried both approaches. As will be seen later, only the second approach was successful, allowing the identification of some amino acid residues involved in the interaction.

Results

The complete high resolution proton n.m.r. spectrum of IF1 is presented in Figure 1A and B. Since a detailed n.m.r. study of IF1 will be presented elsewhere (Paci *et al.*, in preparation), only a brief outline of the main features of the spectrum is presented here. To allow a better evaluation of the spectra presented below, the amino acid composition of IF1 derived from its primary sequence (Pon *et al.*, 1979) is presented in Table I. The major assignable resonances (or groups of resonances) are indicated in the figures by the numbers above the peaks. In particular, in the aromatic region (Figure 1A), one can recognize the resonance of the C-2 protons of the two histidines (His A and His B), the multiple resonances of the protons of a phenylalanine partially restricted in its rotation and that of another freely rotating phenylalanine. At higher fields, the 2,6 and 3,5 protons of tyrosine B and tyrosine A are resolved between 7.2 and 6.8 p.p.m.; partly overlapping these resonances at the pH of this experiment are the two C-4 proton resonances of both His residues. Characteristics of the aliphatic region (Figure 1B)

Table I. Amino acid composition of IF1[a]

Amino acid		Amino acid	
Asp	3	Met	3
Asn	4	Ile	5
Thr	7	Leu	6
Ser	3	Tyr	2
Glu	6	Phe	2
Gln	1	His	2
Pro	2	Lys	5
Gly	5	Arg	6
Ala	2	Cys	0
Val	7	Trp	0

[a]Data taken from the primary structure (Pon *et al.*, 1979).

are the structured resonances of the three serine β-CH_2 between 4.0 and 3.8 p.p.m., the Arg δ-CH_2 (3.25 p.p.m.) and Lys ϵ-CH_2 (3.05 p.p.m.) resonances. Between 1.0 and 0.3 p.p.m., a complex pattern of aliphatic methyl resonances as well as ring current upfield shifted methyl (RCSM) resonances are visible. Overall, the n.m.r. spectrum of IF1 displays a moderate degree of resonance multiplicities and relatively narrow spectral lines, thus suggesting for this protein a rather simple structure endowed with a high degree of flexibility with more or less free aromatic residues.

Upon addition of the highest possible amount of 30S (d) ribosomal subunits so as to reach a 30S:IF1 molar ratio of

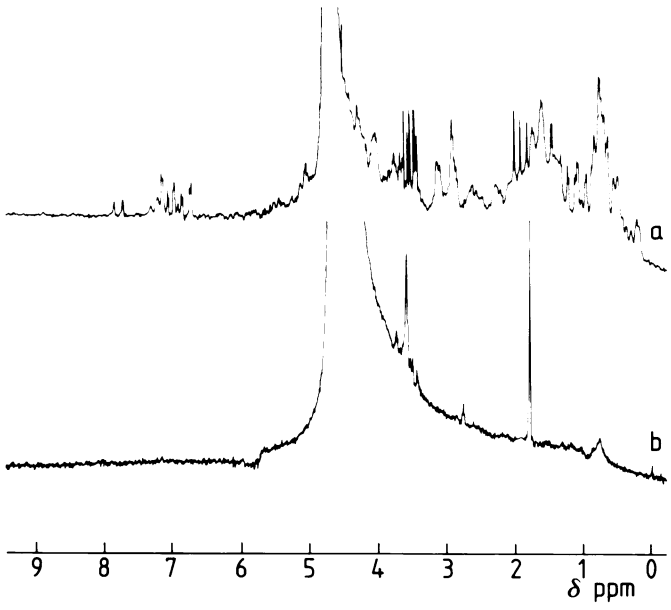

Fig. 2. Effect of 0.25 equivalents of 30S (d) ribosomal subunits on the 400 MHz ^{1}H-n.m.r. spectrum of IF1 **(a)** IF1 alone (2.4 x 10^{-4} M); **(b)** IF1 in the presence of 30S (d) ribosomal subunits. KCl concentration in the buffer was 300 mM. The number of scans was 3000.

0.25, the resonance lines of the factor broaden beyond detection (cf., Figure 2, spectra a and b). Since the resonances due to preparation impurities still display relatively narrow lines, the disappearance of the IF1 spectrum upon addition of the ribosomal subunits cannot be attributed to either a loss of magnetic homogeneity of the sample or other non-specific reasons, such as an increased viscosity. Instead, the selective broadening of all IF1 resonances must be due to an interaction between IF1 and 30S subunits. Indeed, if one takes into account the dissociation constant of the IF1-30S complex estimated under similar conditions from the Airfuge experiments ($K_d \cong 10^{-6}$ M), the relative concentrations of IF1 and 30S in the sample and the estimated relaxation rates of free and 30S-bound IF1 (assuming the same degree of internal mobility in the two species), then the disappearance of the IF1 resonances is to be expected as a result of exchange dynamics between a free and bound form of the factor displaying large differences in line width.

In the light of the above, we used a different approach consisting of the gradual addition of minute amounts of deuterated subunits to a fixed amount of IF1 so as to increase progressively the ribosome:IF1 molar ratio while maintaining a largely sub-stoichiometric concentration. This approach allowed us to monitor small changes of line width and chemical shift before the flattening of the spectra. The observed changes in line width could result from a distribution of chemical shifts resulting from the multiplicity of environments (due to different binding modes) and/or from changes in the individual relaxation rates ($1/T_2^*$) which, assuming a negligible contribution of chemical shift anisotropy, are entirely due to dipole-dipole interactions. In either case, the line broadening is the result of exchange dynamics between free and bound factor having an 'intermediate-fast' rate. Since temperature increases cause distinct narrowing of the broadened resonance lines, it appears unlikely that distribution of chemical shifts is the main reason for the observed line broadenings. Whatever the physical basis of the changes produced by the binding, the selective line broadening and chemical shift alterations, seen against the background of a gradual disappearance of the whole spectrum, allowed us to identify at least some of the amino acid residues of the factor more strongly implicated in and/or affected by the binding. Variations in temperature, which promote changes in mobility and rate of exchange, and increase in ionic strength, which shifts the equilibrium towards the dissociation of the 30S-IF1 complex, also proved useful for studying the interaction.

The effect of the progressive addition of deuterated 30S ribosomal subunits (up to 0.05 equivalents) to a fixed amount of IF1 is shown in Figure 3. As seen in the figure, increasing the amount of 30S (d) (spectra 1 – 7) also causes, in addition to a general broadening of all the IF1 resonance lines, a few selective effects. The aromatic resonances appearing more affected than the others include the C-2 resonances of both His A and His B, and those of one of the two Phe residues (Figure 3A). Concerning the His residues, both C-2 proton resonances are shifted to lower field and both appear broadened. The change in chemical shift, however, is greater for His A than for His B. The line broadening, on the other hand, is greater for His B than for His A. The changes in chemical shift are probably due to local pH variation caused by the polyelectrolyte nature of the 30S ribosomal subunit with which IF1 interacts. In turn, this pH variation could either be selectively greater for His A than for His B, thus implying a more direct involvement of the former residue in an electrostatic interaction with the rRNA or, alternatively, be similar for both residues but cause a differential effect because of pK differences between the two residues (Paci *et al.*, in preparation).

In native free IF1, the two C-2 His protons display virtually equal linewidths. As seen above, however, upon addition of 30S ribosomal subunits, the increase in linewidth is much greater for His B than for His A. Upon addition of 50S ribosomal subunits, a similar but smaller increase in linewidth is also observed indicating that an interaction, albeit weaker, occurs between the factor and the large ribosomal subunit. The plot shown in Figure 4 shows the relative increase of the linewidth of His A and His B C-2 resonances as a function of the molar fraction of either 30S or 50S ribosomal subunits added.

Two selective effects can be quantitatively recognized in the figure. First, the increase in relative linewidth ($\Delta\nu_{1/2}$), induced by the addition of 30S subunits, is ~2.5 times greater for His B than for His A. This suggests that the interaction either involves His B only or involves both His A and His B but with different mechanisms. Second, the line broadening induced by the addition of 30S subunits is much greater than that caused by the 50S subunits. The increase is much greater for His B (≅40%) than for His A (≅20%), thus providing another indication for the selective involvement of the former residue. Furthermore, if the line broadening of His A were entirely due to the dipole broadening caused by the increase in correlation time (τ_c) of IF1 brought about by the binding to large particles such as the ribosomal subunits, then one would expect the relative broadening of His A to be two times greater with the 50S subunits (particle weight ≅1.8 x 10^6) than with the 30S subunits (particle weight ≅0.9 x 10^6) if the IF1-30S and IF1-50S complexes had very similar dissociation constants. On the contrary, the data presented in the figure show the line broadening to be larger with 30S than with 50S

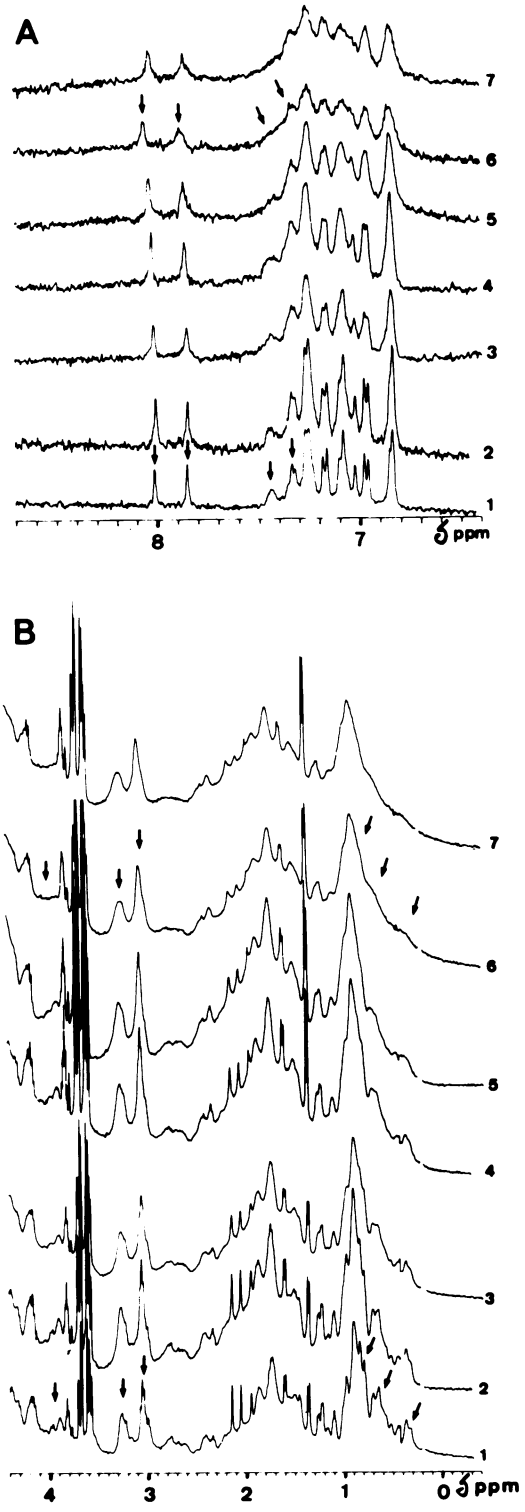

Fig. 3. Effect of increasing amounts of 30S (d) ribosomal subunits on the aromatic **(A)** and aliphatic **(B)** regions of the 400 MHz ^{1}H-n.m.r. spectrum of IF1. The spectral conditions were as described in Materials and methods and the buffer contained 300 mM KCl. **Spectrum 1**, IF1 alone at 4×10^{-4} M; **Spectra 2–7**, 30S (d):IF1 molar ratios of 0.005, 0.01, 0.02, 0.03, 0.04 and 0.05. Approximately 1000 transients per spectrum were recorded. The arrows indicate the resonances undergoing major changes.

subunits. Thus, if His A were not specifically involved, our data would indicate that K_{d50S} is at least one order of magnitude higher than K_{d30S}. This is in good agreement with that previously found by the airfuge binding experiments.

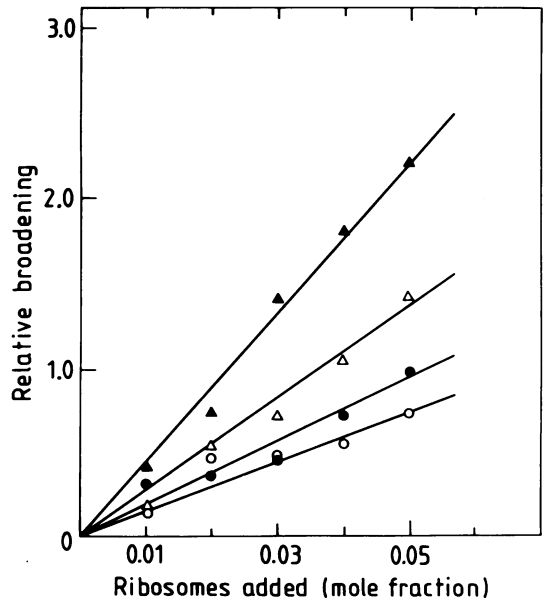

Fig. 4. Effect of increasing amounts of 30S (d) and 50S (d) ribosomal subunits on the linewidths of His A and His B C-2 protons of IF1. The conditions were identical to those described in Figure 3. His A C-2 protons in the presence of 30S (d) (●) or 50S (d) (○) ribosomal subunits. His B C-2 protons in the presence of 30S (d) (▲) or 50S (d) (△) ribosomal subunits.

Of the two Phe residues, that displaying a restricted mobility (Phe A) becomes shifted to higher field and tends to lose its multiplicity (Figure 3A). Thus, upon interaction with the 30S ribosomal subunit, the environment about this phenylalanine changes and this residue tends to acquire a motional behavior similar to that of the other phenylalanine.

As far as the Tyr residues are concerned, it can be seen from the figure that the proton resonances of Tyr B are little influenced by the addition of 30S (d), their resonance lines remaining narrow in spite of the merging of the C-4 proton resonances of His B. The second tyrosine (Tyr A) appears even less influenced by the addition of 30S.

Among the aliphatic resonances more specifically affected by the interaction of the factor with the 30S ribosomal subunits are those assigned to protons belonging to Ser, Arg and Lys residues as well as a number of RCSM resonances (Figure 3B). In particular, one can see that the structured β-CH_2 bands of the three Ser residues (between 4.0 and 3.8 p.p.m.) progressively disappear, while the two nearby peaks remain virtually unaffected. The resonance lines of lysine and, even more so, those of arginines appear broadened and the center of the Arg band is clearly shifted to higher field. Also, the bands due to the methyl groups undergo a marked broadening, especially the RCSM resonances, which are greatly reduced in intensity. In addition to the above effects, shifts and extensive line broadenings can also be detected between 2.9 and 1.0 p.p.m.; the difficulty in assigning resonances in this region, however, prevents us for the moment from drawing more precise indications concerning the residues implicated in these changes. On the other hand, some resonances in the same region (e.g., the three characteristic resonances of S-CH_3 methionine between 1.9 and 2.2 p.p.m.) are little influenced by the interaction and remain rather sharp, indicating a substantial mobility of these groups also in the bound form.

As mentioned above, changes of temperature and ionic

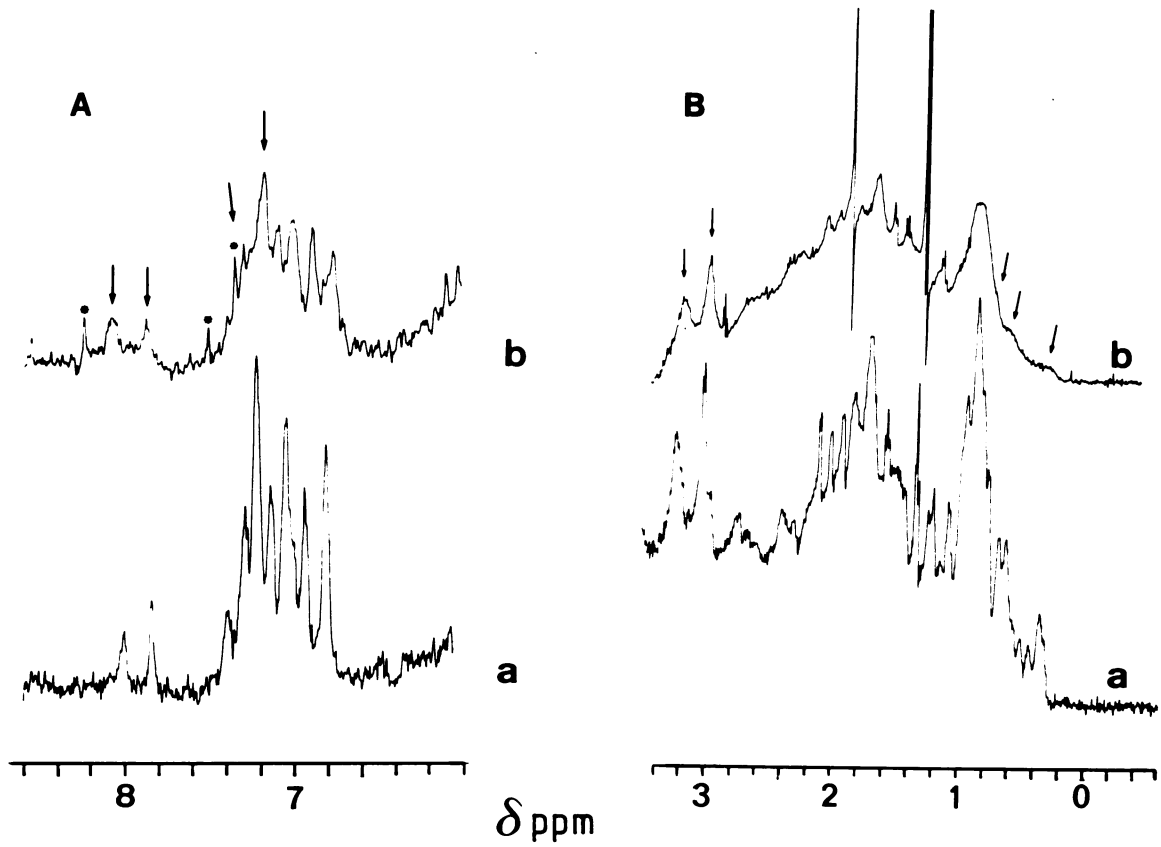

Fig. 5. Effect of 0.025 equivalents of 30S (d) at lower ionic strength on the aromatic (**A**) and aliphatic (**B**) regions of the ^{1}H-n.m.r. spectrum of IF1. Spectral conditions were similar to those of Figure 3 except for the KCl concentration which was 100 mM. (**a**) IF1 alone; (**b**) IF1 in the presence of 30S (d) ribosomal subunits. The arrows indicate the resonances undergoing major changes and the asterisks indicate resonances due to impurities or instrumental artifacts.

strength were made in order to influence the rate of exchange between free and bound factor as well as the binding constant of the IF1-30S complex. Both approaches provided further evidence for the premise that the IF1 spectral alterations observed upon addition of 30S ribosomal subunits are indeed due to a molecular interaction between the two components. In fact, although substantial temperature increases were not possible because of the thermal lability of the samples, moderate temperature increases resulted in a partial reversal of the line broadening effects (not shown). Similar reversals were also observed upon increasing the concentration of monovalent cations and far greater effects on the IF1 spectra were seen, for a given 30S-IF1 molar ratio, depending upon the ionic strength.

In Figure 5, we present the enlarged spectra of the aromatic (Figure 5A) and aliphatic (Figure 5B) regions of IF1, alone and in the presence of 0.025 equivalents of 30S (d) ribosomal subunits in a buffer identical to that used for the experiment of Figure 3 but for the reduction of KCl concentration from 300 to 100 mM. As seen in the figure, all the effects observed in the experiment of Figure 3 at the highest concentrations of added 30S are easily discernible in these spectra, in spite of the much lower concentration of 30S ribosomal subunits present.

Discussion

The results presented here allow the identification of some amino acid residues of IF1 involved in, or affected by, the binding of this factor to the 30S ribosomal subunit. These are His, Arg, Ser, Lys and Phe. The nature of these residues strongly suggests that the interaction between IF1 and 30S subunit has primarily a hydrophilic and electrostatic character, as also suggested by other circumstantial evidence (see Introduction).

The selective disappearance of the β-CH_2 resonances of the Ser residues and the shift to higher field of some of the Arg δ-CH_2 resonances indicated by the large displacement, in the same direction, of the entire Arg band, strongly suggest an interaction involving these residues. From the extent of the changes induced by the binding, it appears likely that all three Ser and more than one of the six Arg residues are implicated. In turn, these interactions could entail either H-bonding or, in the case of the Arg residues, an ionic interaction with the phosphate backbone of the 16S rRNA. A similar interaction can also be postulated for the Lys residues. In this case, however, the overall effect is rather modest so that the involvement of more than one of the five Lys residues appears unlikely. Another effect of the interaction of IF1 with 30S ribosomal subunits is to increase the mobility of a phenylalanine ring previously restricted in its rotational motion. At the same time, a number of RCSM resonances broaden and decrease in intensity indicating that the spatial distribution of these groups is altered by the interaction. Since nuclear Overhauser effect (NOE) experiments indicated the proximity of at least some of the RCSM resonances to this Phe ring (Paci *et al.*, in preparation), it is likely that the two phenomena observed are, at least in part, related. Thus, it would appear that the effect of the binding on the Phe mobility is an indirect one. As to the effects on RCSM

resonances, the present data do not allow us to establish if and to what extent these effects are directly or indirectly related to the IF1-30S interaction.

Both His C-2 proton resonances undergo changes of chemical shift and broaden upon addition of ribosomal subunits. The changes of chemical shift, however, may not be taken as absolute evidence for the participation of these residues in the binding. In fact, small variations of the local pH caused by the polyelectrolyte nature of the ribosome could cause sizeable variations of the chemical shift of these resonances, since our experiments were carried out at physiological pH values which are very close to the pKs of the His imidazole protons. The increase in linewidth, on the other hand, can be unequivocally attributed to a specific interaction of His B with the 30S ribosomal subunits. In fact, the line broadening of the C-2 proton resonance of this residue was found to be markedly greater than that seen for His A and greater for His B in the presence of 30S than in the presence of 50S subunits. These results show that His B is implicated in the binding, while His A is either not involved, less involved or involved *via* a different mechanism. Furthermore, the differences observed in the presence of 30S and 50S indicate that either the binding of IF1 to 30S and to 50S involves similar non-specific effects on the mobility of His A with about two orders of magnitude difference in the binding constants or, if the difference in binding constant is not so high, that only the interaction with the 30S subunits, selectively affects the rotational freedom of the imidazole ring of this residue.

In conclusion, the n.m.r. spectra show that one of the two histidines of IF1 is definitely involved in the interaction with the 30S ribosomal subunit, while the participation of the second His is less certain and its implication in the binding awaits further experiments.

The spectroscopic approach used here complements and extends other studies, carried out in our laboratory, aimed at the identification of the active site of IF1 by means of various selective chemical and/or enzymatic modifications. Though not yet complete, these data can be meaningfully compared with the present ones to give a more comprehensive picture of the nature of the interaction between IF1 and ribosomes. The effect of Arg modification is presently under investigation, while no data is available concerning the serine and phenylalanine residues, since the selective modification of these amino acids is difficult to achieve. On the other hand, in full agreement with that found here, it was shown that the modification of a Lys residue and of one or both His residues inhibits the binding of IF1 to the 30S subunits (Gualerzi and Pon, 1981). Some discrepancies seem to exist concerning the role of the two Tyr residues. In fact, while several chemical properties of these residues appear similar in the n.m.r. and chemical modification studies, the data concerning their implication in the binding are conflicting. Thus, on the one hand, very little effect on the resonances of these residues was observed, while it was found that the enzymatic or chemical iodination of these residues completely abolishes the ribosomal binding and the functional capacity of IF1 (Bruhns, 1980). This discrepancy could stem from the fact that the chemical modification of Tyr induces distortions of the native IF1 folding (e.g., due to the large change of the phenolate pKs) leading to its inactivation. Alternatively, the n.m.r. features of the Tyr residues may remain substantially unchanged through an interaction leaving the overall magnetic microenvironment and mobility of the Tyr residues virtually unaffected.

Materials and methods

Initiation factor IF1 was purified to electrophoretic homogeneity following the previously published procedure (Pawlik *et al.*, 1981). The biological activity of the factor, which was found to be unaffected by the various manipulations required for n.m.r. spectroscopy, was measured as previously described (Pawlik *et al.*, 1981). The factor was concentrated to 1–5 mg/ml ($1.2-6 \times 10^{-4}$ M) and exhaustively dialyzed against several changes of 10 mM potassium phosphate buffer, pH 7.5 (meter reading) containing 100 or 300 mM KCl and 5 mM $MgCl_2$ in D_2O (d > 99%).

High salt washed ribosomal subunits (Risuleo *et al.*, 1976) were obtained from *E. coli* A19 cells grown in ≥80% deuterated medium (a kind gift of R.Lietzke). The deuterated 30S ribosomal subunits used in this study were found to be biologically active (>20 pmol of phenylalanine incorporated per pmol of subunit in standard poly(U)-dependent polyphenylalanine synthesis test). Preliminary experiments showed that these deuterated subunits, 30S (d) and 50S (d), did not give rise to an interfering proton spectrum when subjected to 1000–2000 transients at 400 MHz at $\sim 10^{-5}$ M.

^{1}H-n.m.r. spectra were recorded on a Bruker WM-400 spectrometer operating at 400 MHz with 4 μs (≅30°) pulse, 6000 Hz spectral width and relaxation delay of 1 s. Magnetization decays obtained in the quadrature detection mode were accumulated on 16 k of memory and sensitivity enhancement of 0.8–1.0 Hz was applied before the FT transformation.

Probe temperature was held at 19°C. Samples were analyzed in standard 5 mm tubes. HDO was partially suppressed using an inverse gated pulsed irradiation technique for a duration of 0.6 s.

Half-height linewidths ($\Delta\nu_{1/2}$) were measured in the enlarged spectra obtained without applying exponential filtering.

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