THE MOLECULAR BASIS OF KIRROMYCIN (MOCIMYCIN) ACTION; A ^{1}H NMR STUDY USING DEUTERATED ELONGATION FACTOR Tu

JILL BARBER, JOHN A. CARVER†, REUBEN LEBERMAN†† and GRAHAM M. V. TEBB†

Department of Pharmacy, University of Manchester, Manchester M13 9PL, UK
†Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK
††EMBL Outstation, BP156X, F-38042 Grenoble Cedex, France

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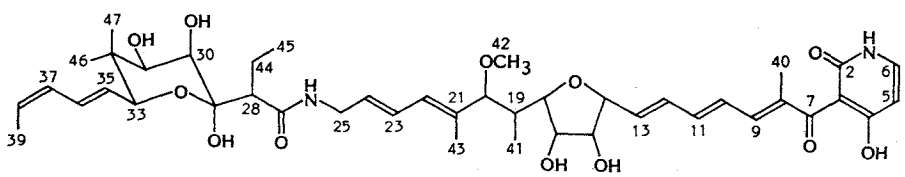
The binding of the antibiotic kirromycin (mocimycin) to its target protein, bacterial elongation factor Tu (EF-Tu), has been studied by ^{1}H NMR spectroscopy using deuterated protein. Narrow lines were observed in the spectrum of the unbound protein (due to residual protons) and in the spectrum of the kirromycin-EF-Tu complex. The spectrum of the complex has been compared with the spectra of the unbound protein and the unbound drug, and the results are interpreted in terms of the mode of antibiotic action of kirromycin.

The bacterial elongation factor Tu (EF-Tu) is a monomeric, acidic, very soluble protein with MW 43,225 in *Escherichia coli*. During elongation of the polypeptide chain in protein biosynthesis EF-Tu forms a complex with GTP and aminoacyl tRNA. This complex binds to the mRNA-ribosome complex, GTP is hydrolyzed and EF-Tu·GDP leaves the ribosome. Kirromycin (**1**), also known as mocimycin, is an antibiotic which binds tightly and specifically to EF-Tu both *in vivo* and *in vitro*. The antibiotic is able to bind free EF-Tu, EF-Tu·GTP and EF-Tu·GDP, the half-life of the complex with this species being about 10 minutes[1]. *In vivo* the antibiotic prevents EF-Tu·GDP from leaving the ribosome and thus inhibits bacterial protein synthesis. Little is known about the molecular basis of kirromycin binding, although the C(18)-C(24) region seems to be important[2]. Cross-linking has been found between kirromycin and lysine 357 of EF-Tu[3], and Schiff's base formation with the carbonyl C(7) has been postulated.

NMR has been used to study the downfield resonances of EF-Tu from *E. coli*[4], and from *Thermus thermophilus*[5], but the remainder of the spectra were broad and unresolved. The effect of deuteration in reducing ^{1}H-^{1}H dipole interactions and hence line widths in the spectrum of EF-Tu has been demonstrated[6]. Deuteration has been used in the study of protein-protein interactions as a means of rendering one part of the complex transparent to NMR[7]. We now describe an examination of



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drug-protein binding by NMR using deuterated protein.

Materials and Methods

Deuterated EF-Tu·GDP was prepared from *E. coli*, MRE 600 as previously described[6], and was stored at −16°C as an ammonium sulfate precipitate. For NMR measurements the precipitate was dissolved in phosphate buffer (60 mM sodium phosphate, 5 mM magnesium chloride, 0.5 mM dithioerythritol in D_2O, apparent pH 7.25) and dialyzed three times against the buffer. The protein in the final solution was found by BRADFORD's method[8] to be 80 mg/ml. EF-Tu exists in the cytosol as a complex with GDP. The dissociation constant for this complex is of the order of 10^{-9} M so there is negligible loss of GDP during the isolation of the protein and its preparation for ^1H NMR experiments. This is clearly also the form of the protein to which kirromycin binds *in vivo*.

Mocimycin (kirromycin) was a gift from Dr. R. BEUKERS (Gist-Brocades n.v., Delft, Holland), and was purified by his method (personal communication). The pure antibiotic was stored at −16°C.

The ^1H spectrum of kirromycin in acetone-d_6 has been completely assigned by two-dimensional (2D) correlation spectroscopy (J. BARBER, A. E. DEROME and G. M. V. TEBB; unpublished results) and the spectrum in buffer was assigned by comparison with this spectrum and with spectra recorded in mixed solvent systems.

^1H NMR measurements were recorded in a 5-mm sample tube using a Bruker WH-300 NMR spectrometer operating at 300 MHz. The water signal was suppressed by a selective presaturation pulse of 1 second duration, and the temperature was maintained at 297°K. The resolution was enhanced using a Gaussian window function. The spectra were referenced to sodium 3-(trimethylsilyl)-propionate (TSP) as internal standard. The initial sample contained 40 mg protein in 0.5 ml buffer. The effect of kirromycin was observed by adding successive 2 μl portions of a 1/20-M solution in MeOH-d_4 up to a total of 40 μl (2 equivalents approx). Each spectrum was accumulated for 1,000~1,500 scans.

A spectrum of unbound EF-Tu·GDP in the presence of approximately one equivalent of unbound kirromycin was simulated by spectral addition in order to facilitate interpretation of the spectra of the complex.

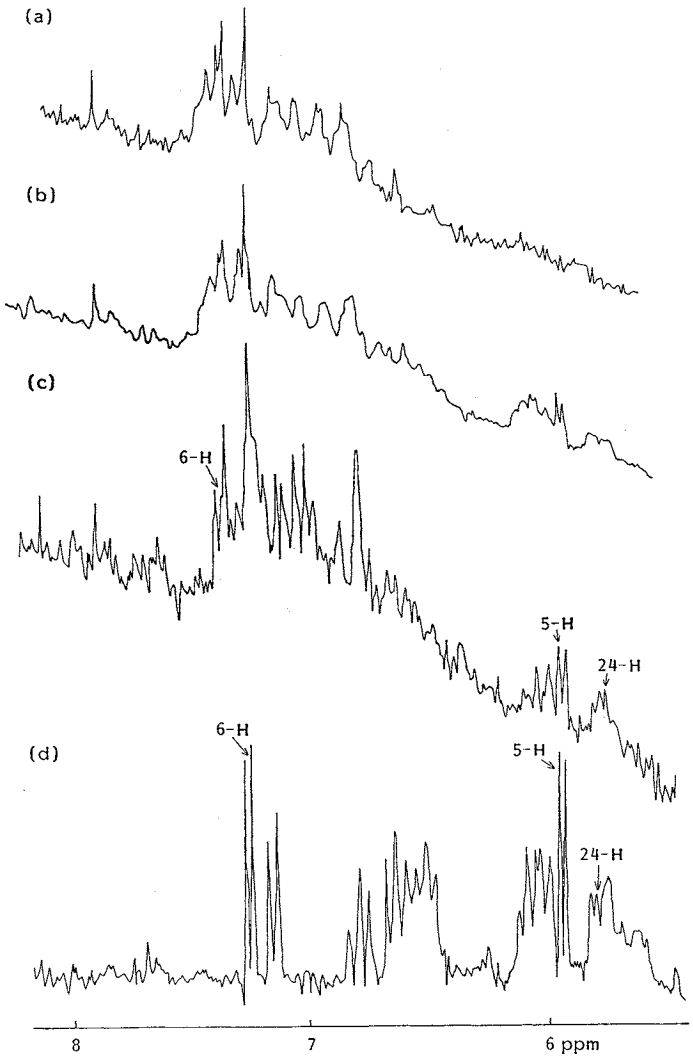
Results and Discussion

At 90% deuteration the residual signals from EF-Tu were still quite strong, and the protein gave rise to a spectrum consisting mainly of narrow lines as shown previously[6]. Gradual addition of kirromycin to the protein solution produced marked changes up to the point where 1 equivalent of kirromycin had been added, after which there was little change until a large excess had been added and the spectrum of free kirromycin was discernible. Thus by monitoring regions of the spectrum in which major changes occur on binding, for example the aromatic region (Fig. 1) and the aliphatic methyl region (Fig. 2), it was possible to confirm that, when one equivalent of kirromycin had been added, the only species detected by NMR was the 1 : 1 complex, as expected from the reported dissociation constant of 10^{-7}~10^{-8} M[8].

The spectrum of the 1 : 1 complex was compared with the simulated spectrum, equivalent to a 1 : 1 mixture of unbound kirromycin and protein. Substantial differences are apparent in all regions of the spectra but are particularly clear in the two regions of the spectra shown (Figs. 1 and 2). This result is consistent with kirromycin inducing a major conformational change in the protein on binding and with previous suggestions[9,10] that when EF-Tu·GDP binds to kirromycin a conformation which resembles, but is not identical with, the conformation of EF-Tu·GTP is adopted.

Application of the rigid rotor nearest-neighbor (RRNN) model for relaxation, assuming that the diffusional correlation time for the kirromycin-EF-Tu·GDP complex is about 10^{-8} second and that

Fig. 1. ^{1}H NMR spectra; aromatic region.
(a) EF-Tu·GDP, (b) simulated 1 : 1 kirromycin: EF-Tu·GDP, (c) kirromycin-EF-Tu·GDP complex, (d) kirromycin. Not to scale.

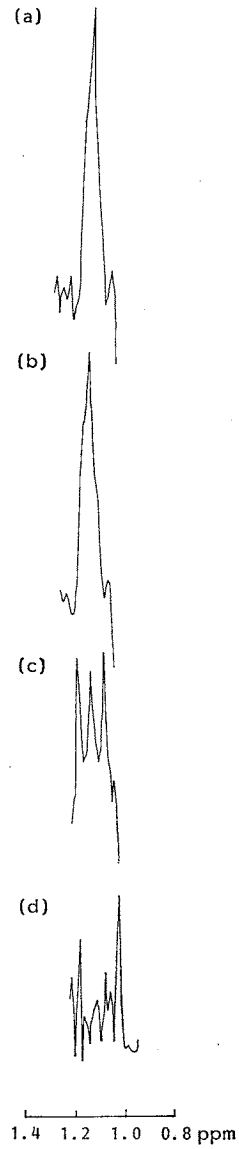


^{1}H-^{1}H dipole-dipole interaction is the principle mode of relaxation, suggests that line widths for resonances due to the alkene portions of kirromycin in the drug-protein complex should be about 4~5 Hz if free rotation is not permitted; the line widths of the methyl group resonances are predicted to be in the region of 30 Hz (assuming complete immobilization; naturally the lines will be much sharper if spinning about the symmetry axis is permitted). The aromatic and alkene regions of the spectrum of the 1 : 1 antibiotic-protein complex (Fig. 1) show narrow lines due to both the drug and the protein, as expected. Closer examination reveals that the doublet due to 5-H of kirromycin is essentially unaffected by binding to EF-Tu. Irradiation in the region δ 7.0~7.5 gave a maximum decoupling effect very close to δ 7.29, the position of the signal due to 6-H (which couples to 5-H), in the spectrum of free kirromycin. Changes are observed, however, in the multiplets on either side of the 5-H doublet. The upfield multiplet is due to 13-H, 22-H and 37-H, the downfield multiplet to 24-H, 35-H and 38-H.

It is not possible to interpret these changes in detail but the signal due to 24-H appears to be upfield shifted by about 0.05 ppm.

These results strongly imply that the pyridone ring of kirromycin is not intimately involved in the binding of the drug to EF-Tu and are consistent with the observation that a number of active analogues of kirromycin are modified in this ring[8]. These findings are also consistent with results obtained from studying the binding of semisynthetic analogues of kirromycin to EF-Tu, which suggest that the alkene region C(18)-C(24) is involved in binding[2]. UV spectroscopy suggests that the part of the molecule containing the chromophore N(1)-C(13) is affected by binding[1], and is located in a hydrophobic region of the protein, and our results are consistent with C(7)-C(13) being involved in binding.

Fig. 2. ^{1}H NMR spectra; aliphatic methyl region.
(a) EF-Tu·GDP, (b) simulated 1 : 1 kirromycin: EF-Tu·GDP, (c) kirromycin-EF-Tu·GDP complex, (d) kirromycin. Not to scale.



Conclusion

The use of deuterated protein has allowed NMR studies to be carried out on a tightly-bound, relatively large drug-protein complex. Information of potential importance in drug design has been obtained using a simple 1D experiment at 300 MHz; refinements such as the use of higher spectrometer frequency, 2D experiments and higher levels of deuteration in the protein are expected to yield more detailed results. The use of deuterated protein complements other NMR methods used in drug design[11,12], and will be of particular value in the study of tightly-bound complexes and complexes involving thermally unstable proteins.

Acknowledgments

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