Tautomeric Recognition of Erythromycin A by Ribosomes: A ^{1}H Nuclear Magnetic Resonance Study

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^{1}H NMR spectroscopy has been used to demonstrate specific binding of bacterial ribosomes to the keto tautomer of erythromycin A in a fast exchange process.

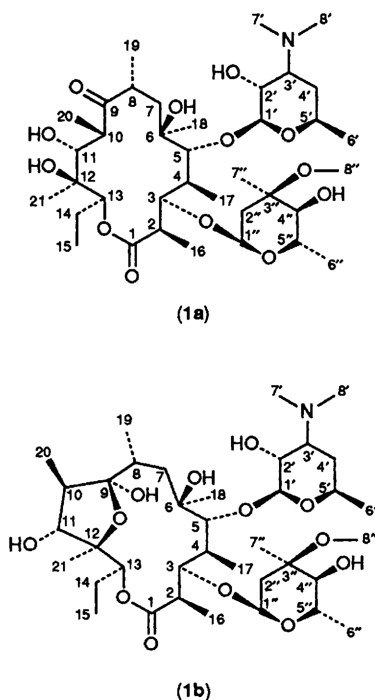
The macrolide antibiotic erythromycin A (**1**) acts on the peptidyl transferase centre of the bacterial ribosome, so inhibiting protein synthesis. It is believed that both sugar residues are required for maximum drug activity,[1] and that the large ribosomal subunit proteins L15 and L16 are important in the binding of erythromycin to ribosomes.[2] Erythromycin A is reported to be inactive below pH 6.9.[1]

In aqueous solution erythromycin A exists as an equilibrium mixture of the 9-ketone (**1a**) and, as we have recently shown,[3] the 12—9 cyclised hemiketal (**1b**). At ambient temperature and physiological pH the keto form is the more abundant, existing in a 2.5 : 1 mixture with the hemiketal. The ^{1}H and ^{13}C NMR spectra of the ketone in $CDCl_3$ solution have been fully assigned,[4] and we have largely assigned the ^{1}H and ^{13}C NMR spectra of both forms in aqueous solution at pH 7.1.[3]

The present experiment was prompted by the work of Tritton,[5] who observed differential line broadening (due to fast exchange between bound and free drug) in the ^{1}H NMR spectrum of chloramphenicol on titration of ribosomes into a large excess of the drug. It was concluded that the more extensively broadened signals were due to those parts of the drug which bind directly to the ribosome. We have carried out a similar experiment in an attempt to gain information about the molecular basis of the binding of erythromycin A to ribosomes.

To a 4 mM solution of erythromycin A (recrystallised twice from chloroform–hexane) in deuteriated buffer (50 mM sodium phosphate, 200 mM KCl at pH 7.6) were added successive aliquots of a solution of washed *E. coli* ribosomes in the same buffer to a final concentration of 12 μM and the 500 MHz ^{1}H NMR spectrum was recorded. Finally the pH was adjusted and spectra run with the sample at pH 8.5, 9.4, and 6.2. In another experiment erythromycin A (4 mM) was treated with increasing concentrations of ribosomal 'core' particles to a final concentration of 19.5 μM, and the ^{1}H NMR spectrum run at pH 7.6. The 'core' particles are obtained by treating 50 S ribosomal subunits with 1.3 M lithium chloride solution; this removes a number of the proteins, including L15 and L16 which are required for specific erythromycin binding.[2] In terms of mass per unit volume, 19.5 μM 'core' particles correspond approximately to 12 μM ribosomes. All experiments were repeated at least twice with slight variations in absolute linewidths (due to differences in shimming, for example). The trends reported here, were, however, completely reproducible.

Figure 1 shows the ^{1}H NMR spectra of erythromycin A, erythromycin and core particles, and erythromycin and ribosomes. Figure 2 shows expansions of the region δ 0.7—1.0 containing the upfield triplets due to H_3-15. All the spectra were processed without weighting. The effect of the core particles was to broaden the whole spectrum somewhat. The erythromycin A spectrum is complex but the linewidths of 19



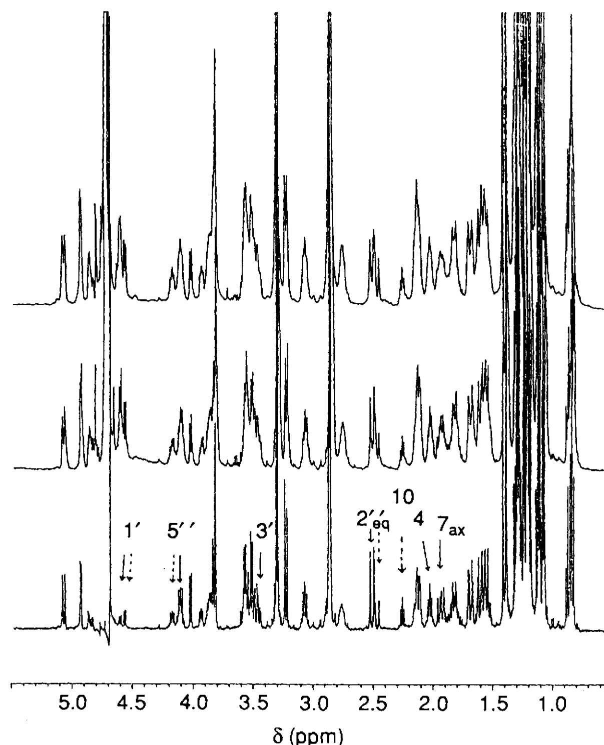


Figure 1. 500 MHz ^{1}H NMR spectra of 4 mM erythromycin A in deuteriated sodium phosphate buffer: A at pH 7.6; B in the presence of 19.5 μM ribosomal core particles at pH 7.6; C in the presence of 12 μM ribosomes at pH 7.6.

Table 1. Linewidths at half height in the ^{1}H NMR spectrum of erythromycin A alone and in the presence of ribosomes; selected signals from major and minor tautomers.

	δ (ppm)		Linewidths at half height (Hz) in the spectrum of erythromycin A					
			pH 7.6		With 19.5 μM cores pH 7.6		With 12 μM ribosomes pH 7.6	
Position	Major	Minor	Major	Minor	Major	Minor	Major	Minor
1″	4.93	4.87	2.75	3.25	4.9	4.9	5.5	5.1
$2''_{eq}$	2.51	2.48	2.75	2.5	4.4	4.25	8.6	5.25
5″	4.15	4.20	2.5	2.5	3.6	3.4	12.25	5.1
8″	3.31	3.38	1.5	1.25	2.6	3.5	8.6	5.6
1′	4.61	4.55	2.5	2.5	3.5	3.1	8.5	5.1
3′	3.48	—	2.0	—	5.0	—	11.9	—
7′,8′	2.85	2.84	2.1	2.25	3.25	3.75	5.15	5.25
7_{ax}	1.94	—	3.75	—	4.9	—	12.5	—
15	0.84	0.88	1.9	1.55	3.75	3.1	7.0	5.1
17	1.08	1.19	2.6	2.4	4.0	4.9	9.0	7.5
18	1.37	—	2.4	—	4.4	—	7.4	—
21	1.21	1.42	2.25	2.1	4.0	3.75	12.0	5.0

signals due to the major tautomer and 13 due to the minor tautomer could be distinguished.† These signals had a half-height line width on average 1.7 times that of the corresponding signal in the spectrum of erythromycin alone. The standard deviation from this average was only 0.35, and there was no discernible difference between the major and minor tautomers. This indicates that the only interactions between erythromycin and cores are non-specific and typical of weak interactions between small molecules and large proteins and protein complexes. The effect of whole ribosomes on the spectrum of erythromycin was quite different. Selective line broadening was observed, the signals due to the major tautomer being broadened more than those due to the minor tautomer (see signals due to H-$2''_{eq}$, H-5″, H-1′, and H_3-15), and with much greater variation in the extent of broadening. Table 1 gives linewidths for some representative signals.

It is clear from these data that *E. coli* ribosomes are able to bind erythromycin A, preferentially to the major tautomer, in a fast exchange process. Some lines in the spectrum (for example, those due to H-$2''_{eq}$, H-5″, H_3-21, H-7_{ax}, and H-3′) of the major tautomer are broadened more than others, indicating that this binding is specific. Line broadening may result from two distinct processes, rapid equilibrium between states with different chemical shifts, and slowing of molecular motion. It is difficult to distinguish between these but, in general, a correlation between line broadening and involvement in binding is expected. It is, therefore, tempting to suggest that those parts of the molecule which give rise to the most line broadening, for example, the cladinose ring and the methyl group $C(21)H_3$, are those most closely associated with the ribosome on binding.

In order to consider the effect of pH, 'broadening' (in Hz) was defined as the increase in linewidth in a particular signal in the erythromycin–ribosomes spectrum compared with the same signal in the erythromycin–cores spectrum. The spec-

† The compounds described here are ring-chain tautomers. The term tautomer, rather than isomer, is used to stress that the structures are in equilibrium.

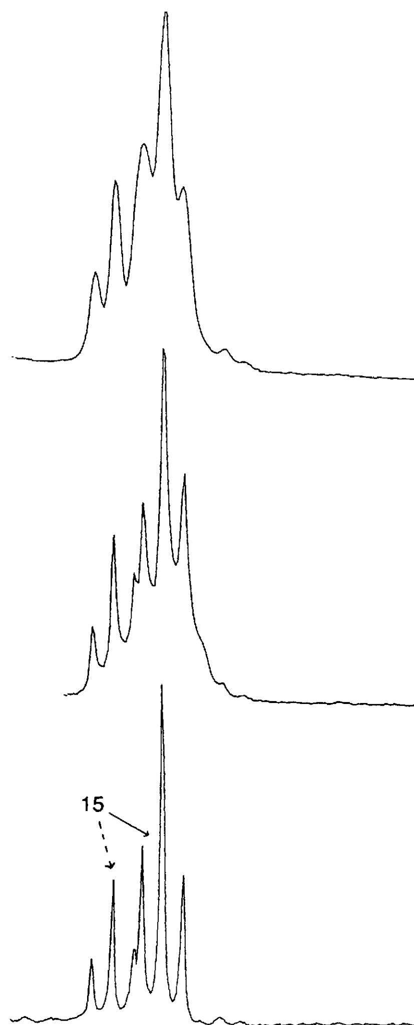


Figure 2. Expansion of Figure 1 (δ 0.7—1.0) showing the triplets due to H_3-15.

trum of erythromycin A alone shows extreme narrowing (under which conditions it is more appropriate to consider the factor by which linewidths are increased than the absolute amounts); in the presence of cores erythromycin A gives a spectrum with lines of similar width to the narrowest lines in the spectrum of the drug bound to ribosomes, and allows the comparison of like with like. At all pH values tested the mean broadening was small for the minor tautomer (0—2 Hz) but larger (4—7 Hz) for the major tautomer, indicating that ribosomes recognise the major tautomer over the whole pH range under study. The difference between the mean broadening for the two tautomers increased with pH (pH 6.2, 2.2 Hz; pH 7.6, 3.7 Hz; pH 8.5, 4.4 Hz; pH 9.4, 5.0 Hz), giving some indication that the binding of the major tautomer is tighter at higher pH. Few of the individual resonances show any marked trends, however, and only for a very few protons are data available for both tautomers over the whole pH range, so this analysis is necessarily crude. Further experiments, such as T_1 measurements, are required to probe the molecular basis of the effect of pH.

The dissociation constant for the erythromycin–ribosome complex has been measured as 10^{-7} M[6] and as 10^{-8} M,[7] values which would suggest that the bound drug should be in slow exchange with the free. In this case the drug is present in large excess over the ribosomes so, were slow exchange being monitored, the spectrum would be essentially that of free erythromycin, broadened slightly and uniformly by non-specific interactions. It seems probable, therefore, that the binding site monitored in this experiment is a second, low affinity site, probably unrelated to peptidyl transferase. Low affinity binding sites for erythromycin have been observed on *Bacillus subtilis* ribosomes.[8] Like the high-affinity site this binding site (or possibly binding sites) may favour higher pH (presumably the erythromycin free base, rather than the protonated form), but the most interesting observation is the tautomeric recognition. Ketone–hemiketal tautomerism in erythromycin is very temperature dependent and solvent dependent but not very pH dependent. In non-polar solvents the hemiketal is hardly observed; in aqueous solution at 50 °C it is present at only 20% total erythromycin.[3] The present study demonstrates that ribosomes are able to distinguish between the tautomers of erythromycin A and that chemical and biochemical studies, in particular kinetic and equilibrium analyses, of erythromycin A will, in addition to being pH sensitive, be very temperature and solvent sensitive.

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