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Solvent spin-labelling for investigating the interaction of biological ligands with macromolecules

A ^{1}H paramagnetic relaxation study

Neri Niccolai [a], Raffaella Lampariello [b], Lucia Bovalini [c], Mauro Rustici [a], Paolo Mascagni [d] and Paola Martelli [c]

[a] *Dipartimento di Chimica,* [b] *Istituto di Chimica Organica and* [c] *Istituto di Chimica Biologica, University of Siena, 53100 Siena, Italy and* [d] *Department of Pharmaceutical Chemistry, University of London, London WC1N 1AX, U.K.*

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The paramagnetic contributions to the spin-lattice relaxation rates of khellin protons, induced by the presence in an aqueous solution of TEMPO nitroxide, have been analyzed in the interaction of the furochromone with DNA. The relaxation data obtained at different temperatures, nitroxide and DNA concentrations indicate that the average solvent exposure of the furanic moiety of khellin is lower than that of the pyranic group. This feature suggests that the former is the main site of approach of khellin to DNA.

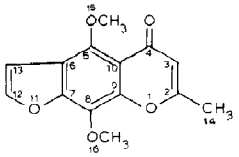
1. Introduction

It has been shown that the localization of solvent-exposed moieties of biopolymers can be achieved by spin-labelling the solvent molecules with free radicals, such as TEMPO nitroxide, and by observing the perturbation induced by the paramagnetic species on the proton spin-lattice relaxation rates of the solute molecules [1,2]. The formation of intermolecular adducts between biological ligands and macromolecules may cause strong changes in the solvent exposure of molecular sites whose proton spin-lattice relaxation behaviour can be observed. Thus, in the present report, the latter paramagnetic relaxation technique has been explored for obtaining information on the mechanisms of the intermolecular interaction of complex species in solution. As a model system, the DNA-khellin interaction is analysed here, since khellin, as well as other furochromones and psoralens, exhibit a strong affinity with nucleic acids [3–6]. The mechanisms of their interaction have been proposed [7] and non-covalent binding between the two molecules has been suggested as a preliminary step for the formation of the covalent photoadducts [8] which are responsible for the phototoxicity and genotoxicity of furochromones [9,10]. The presence in solution of these non-covalent khellin-DNA adducts is investigated in this article by analysing the TEMPO-induced paramagnetic perturbations on proton relaxation of khellin nuclei.

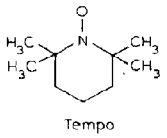
Correspondence address: N. Niccolai, Dipartimento di Chimica, University of Siena, 53100 Siena, Italy.

2. Materials and methods

Khellin, 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) and highly polymerized DNA from calf thymus were obtained from Fluka, Molecular



khellin



Tempo

Structure 1.

Probes and Sigma and used without further purification. Solutions were prepared by dissolving 3.1 mg of khellin in 0.5 ml D_2O containing 20% DMSO-d_6 and 2.1 mg DNA in 10.6 ml D_2O. Paramagnetic solutions were prepared by adding TEMPO to the khellin and DNA solutions to obtain a free radical concentration of 1 mM. For studying the perturbation of khellin solvent exposure due to the presence of DNA, 0.1 ml of the paramagnetic solution of the nucleic acid was added to the khellin diamagnetic and paramagnetic solutions. The same volume of diamagnetic DNA solution was added to khellin in order to observe relaxation variations of the furochromone protons induced by the macromolecule in the absence of the nitroxide. Proton partially relaxed spectra, obtained by using the inversion recovery pulse sequence, were recorded with a Varian XL-200 NMR spectrometer. Relaxation rates were calculated by computer fitting of relaxation curves and the calculated experimental errors were always lower than 5%. The diamagnetic khellin-DNA solution in the D_2O/DMSO-d_6 mixture was investigated by spectrophotometric determinations of the absorbance at 260 nm in the range of temperature used for the NMR study. No hyperchromic shifts, typical of DNA denaturation, were observed at temperatures lower than 70 °C. Molecular models of khellin were generated on a PS 390 Evans & Suterland system; the refinement of the molecular structure used for the calculation of the calibration distance between H_{12} and H_{13} (see section 3) was achieved by using the Tripos force field energy minimization routine of the SYBYL software.

3. Results and discussion

The spin-lattice relaxation rates of khellin protons measured at 25 and 60 °C in the additional presence of TEMPO and DNA are listed in table 1. In the diamagnetic solutions, khellin protons

Table 1

Proton spin-lattice relaxation rates of khellin nuclei in solution at 25 and 60 °C

		δ (ppm) [a]	R_d	R_{dDNA}	R_p	R_{pDNA}	R_{pDNA}/R_p
25 °C	H_{12}	7.70	0.39	0.44	3.99	4.50	1.12
	H_{13}	7.09	0.55	0.60	3.63	3.92	1.08
	H_3	6.00	0.40	0.42	3.61	4.52	1.25
	H_{15}	3.99	0.46	0.58	4.43	4.00	0.90
	H_{16}	3.92	0.55	0.63	3.93	3.71	0.94
	H_{14}	2.27	1.38	1.60	3.48	3.22	0.92
60 °C	H_{12}	7.73	0.08	0.25	1.86	1.78	0.95
	H_{13}	7.07	0.23	0.37	1.61	1.62	1.00
	H_3	6.01	0.09	0.24	1.84	1.89	1.03
	H_{15}	4.00	0.17	0.22	1.79	1.75	0.98
	H_{16}	3.91	0.22	0.26	1.64	1.61	0.98
	H_{14}	2.27	0.52	0.63	1.68	1.56	0.93

[a] Chemical shifts from DMSO residual resonance at 2.48 ppm. Relaxation rates, as defined in the text, are s^{-1}.

exhibit slow relaxation rates, R_d, as expected for such a small molecule, since the molecular motions and the magnetic environments of these proton nuclei are not very suitable for the relaxation process. At higher temperature this behavior is even more apparent since, under this condition, faster correlation times are effective. An estimate of the correlation times which determine the relaxation of proton nuclei attached to their rigid framework can be made by considering the dipolar interaction between H_{12} and H_{13} as the main source of their relaxation. On the basis of eq. 1, a calibration distance of 2.58 Å for the H_{12}–H_{13} internuclear vector and the spin-lattice relaxation rate measured for H_{12}, information on the dynamics of khellin under the various experimental conditions can be obtained, as:

$$R_d = \frac{h^2\gamma^4}{10r^6}\left[\frac{3\tau_c}{1+\omega_0^2\tau_c^2} + \frac{6\tau_c}{1+4\omega_0^2\tau_c^2} + \tau_c\right] \qquad [1]$$

where r denotes the internuclear distance, τ_c is the correlation time and the other terms are characteristic constants of the observed nucleus. Correlation times of 1.4×10^{-10} and 3×10^{-11} s were calculated to be effective for the selected internuclear vector at 25 and 60 °C, respectively. The presence of DNA in the diamagnetic khellin solution causes an increase in the relaxation rates, R_{dDNA}, of all protons at both temperatures (see table 1). The correlation times for the reorientation of the H_{12}–H_{13} internuclear vector in the presence of the nucleic acid are then calculated to be 1.4×10^{-9} and 8×10^{-11} s, respectively, at 25 and 60 °C. It should be noted that the other two solutions of eq. 1, i.e., 1.7×10^{-10} and 3.0×10^{-9} s, respectively, for the two temperatures, are not consistent with the observed motional changes of khellin in the absence of DNA.

Thus, it can be proposed that the molecular motion of the furochromone in water solution satisfies the extreme narrowing condition: $\omega_0\tau_c \ll 1$. The latter condition does not hold at room temperature, when DNA is added to the solution. In fact, the specific intermolecular interactions which occur between the nucleic acid and khellin considerably reduce the reorientation of the rigid framework of khellin.

Table 2

Effective correlation times of khellin interproton vectors of methyl groups

Correlation times, calculated from eq. 1 and a calibration distance of 1.8 Å, are reported in ns. Values in parentheses refer to the temperature of measurement, while the subscript DNA indicates that these correlation times of khellin methyls have been calculated in the presence of the macromolecule.

	$\tau_c(25)$	$\tau_{cDNA}(25)$	$\tau_c(60)$	$\tau_{cDNA}(60)$
H_{15}	0.009	0.010	0.004	0.005
H_{16}	0.011	0.012	0.005	0.005
H_{14}	0.028	0.031	0.010	0.012

The presence of DNA in solution causes smaller changes in the reorientation of geminal interproton vectors of CH_3 groups, since extensive internal motions occur. In fact, assuming for the latter protons a pure dipolar relaxation, eq. 1 can be used to calculate the effective correlation times reported in table 2. For these protons it can be concluded that extreme narrowing conditions hold even in the presence of DNA.

As shown in table 1, the addition of the free radical TEMPO to the solution speeds up the relaxation of all khellin protons at both temperatures. The paramagnetic relaxation contribution, R_p, to the observed spin-lattice relaxation rate, R_{obs}, defining R_p as the difference $R_{obs} - R_d$, provides a measure of the effectiveness of the proton-electron dipolar interaction. The paramagnetic contribution depends on the distance of closest approach between the electronic and nuclear spins and on the correlation time which modulates this dipolar interaction [11]. The calculated R_p values of khellin protons in the presence of TEMPO are very similar and, hence, a random approach of the nitroxide with the furochromone molecules can be proposed and the observed paramagnetic relaxation enhancements can be interpreted in terms of solvent exposure and molecular motion. At 60 °C, all the R_p values are considerably smaller than those measured at 25 °C, due to the presence, at higher temperature, of faster molecular motions which decrease the effectiveness of the dipolar electron-nucleus interaction. The addition of DNA to the khellin-TEMPO solution modifies in different ways the paramagnetic

contributions calculated for methine and methyl protons of the furochromone. The presence of DNA in the solution induces an increase in the relaxation rates of CH protons, while the opposite behaviour is observed for those of CH_3. The analysis of the paramagnetic relaxation contribution R_{pDNA} of khellin protons, defined as the difference between R_{obs} and R_{dDNA}, allows the interpretation of relaxation data in terms of molecular dynamics. The stereospecificity of the intermolecular interaction can be more directly observed by discussing the value of the ratios R_{pDNA}/R_p. The fact that the calculated ratios for H_{12} and H_{13} are similar and considerably smaller than the value found for H_3 clearly indicates that DNA molecules shield from the solvent the furanic moiety more than the remaining part of khellin. These findings are consistent with the presence on the pyranic ring of a methyl group whose steric hindrance may reduce the probability of formation of the collisional adducts via this molecular site. As far as methyl protons are concerned, the previously determined ratios are all found to be similar and less than unity. A generalized solvent shielding of khellin methyls, due to the presence of DNA, can account for the latter ratios. The overall reduction of the paramagnetic relaxation, under the same conditions as when not observed for methine protons, can be ascribed to weaker dipolar couplings between the unpaired electron and methyl protons. This can be due to the occurrence of faster correlation times which determine the extent of the dipolar interaction.

It can be suggested that, in general, information on the mechanisms of the intermolecular interaction involving complex molecular systems can be obtained by spin-labelling the solvent with TEMPO nitroxide and monitoring the selective changes in paramagnetic spin-lattice relaxation rates of well-resolved nuclear resonances. It should be noted that the paramagnetic proton relaxation approach proposed here, seems to be confined to the investigation of the intermolecular interaction of small ligands with macromolecules. Nonetheless, isotopic enrichment and/or nuclear replacement may represent a powerful means of obtaining information on native systems of pharmacological and immunological relevance.

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