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Molecular Recognition

II—Discrimination of Specific and Non-Specific Intermolecular Interactions by Means of Magnetic Resonance Spectroscopy†

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ABSTRACT: The interactions of adenine- and benzimidazole-type substrates with a model receptor (Rebek's cleft) and benzoic acid were studied by magnetic resonance spectroscopy. The spectra reveal dynamic phenomena which are analysed in terms of static, two-jump and two-site models. The interpretation provides information on the inter- and intramolecular structures and allows specific interactions to be distinguished from non-specific interactions.

KEYWORDS: molecular recognition; molecular dynamics; exchange

INTRODUCTION

Particular molecules recognize each other, associate and form lock-and-key complexes. These interactions between receptors and substrates, known collectively as molecular recognition, govern basic principles of life such as regulation, transport and catalysis. If two molecules are able to interact in a specific manner, they are said to be complementary in size and shape. The resulting stereochemistry ensures the proximity and confrontation of functional groups so as to establish attractive forces of electrostatic, hydrogen bonding or van der Waals type.[2] Owing to the non-covalent character of the attractions, lock-and-key relations are usually found to be equilibria depending on environmental parameters.

The study of molecular recognition by spectroscopic methods requires detectable probes at the species to be observed. Concerning magnetic resonance, most organic molecules can be readily investigated by NMR spectroscopy, but for the application of EPR/ENDOR (EMR) spectroscopy at least one of the molecules has to bear a paramagnetic moiety reflecting the changes in the spectroscopic properties caused by any type of interaction.

In the course of our investigations, the molecular cleft **R** proved to be suitable, providing a lock-like cavity with specific affinity to purine and benzimidazole derivatives.[3] We therefore modified these heterocycles with pre-paramagnetic phenol-type substituents (Scheme 1)

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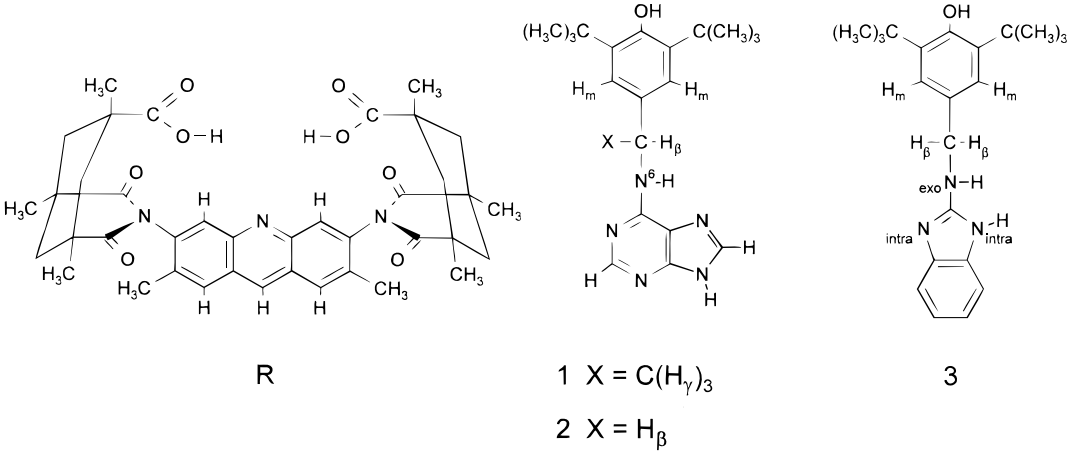

Scheme 1. Receptor **R** and substrates **1**, **2** and **3**.

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in sites where they do not interfere with the intermolecular interactions.[1]

The EMR investigations reveal, owing to the better time resolution compared with 250 MHz NMR, the dynamic behaviour of all components: the global intermolecular equilibrium and the intramolecular flexibility. Hence, different phenomena are traced back to the internal motions: superposition of hyperfine structures and exchange based upon a two-jump mechanism and upon a two-site model.[4] In this paper, we will show that by means of EMR spectroscopy specific lock-and-key interactions can be distinguished from non-specific interactions.

EXPERIMENTAL

The receptor **R** is well known.[5] The syntheses of phenols **1**, **2** and **3** have been described in theses.[6,7]

2,6-Di-*tert*-butyl-4-(dimethylaminomethyl)phenol, lead dioxide, benzoic acid and dichloromethane (CH_2Cl_2) are commercially available. Lead dioxide was further dried *in vacuo*. Benzoic acid was recrystallized from toluene. CH_2Cl_2 was redistilled and stored over molecular sieves.

Solutions of the diamagnetic species in CH_2Cl_2 were prepared containing substrate concentrations between 1 and 10 mM. The radicals **1***, **2*** and **3*** were generated by monovalent oxidation of the corresponding phenols with PbO_2. The receptor **R** or benzoic acid (BA) can be added before or after radical generation according to Scheme 2. Deoxygenation was performed by bubbling argon through the samples.

The spectra were recorded with a Bruker ESP 300E spectrometer equipped with an ENDOR unit (Bruker ER 810). Typical instrumental parameters for ENDOR investigations were microwave power 30 mW, r.f. power 7 dB, 500 W and modulation 70 kHz.

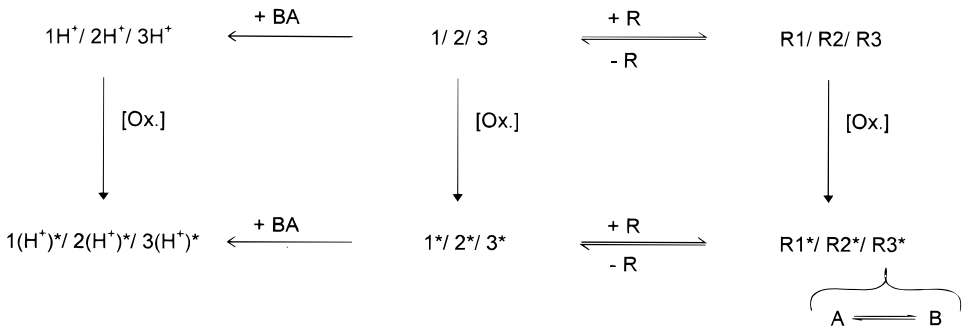
RESULTS

The affinity of **R** towards **1** and **2** has already been investigated quantitatively by ^{1}H NMR spectroscopy in $CDCl_3$ (NMR titration) yielding $K_A = 1 \times 10^3$ l mol^{-1} for **R** + **1** ⇌ **R1** and 3×10^3 l mol^{-1} for **R** + **2** ⇌ **R2**.[1] As the addition of acids has had no significant influence upon the chemical shifts of the substrates **1**, **2** and **3**, the observed chemical shift differences $\Delta\delta = \delta_{compl} - \delta_{free}$ (CIS values[8]) are traced back to complexation.

Hence samples of free radicals **1***, **2*** and **3*** were prepared, together with mixtures with **R** and with benzoic acid, according to Scheme 2.

1* and 1* + R

The phenoxyl **1*** at 232 K presents an EPR spectrum which is readily interpreted in terms of the hyperfine (hf) coupling parameters given in Table 1. Addition of receptor **R** alters the hf structure. The spectrum recorded at a substrate–receptor ratio of 1:1 is presented in Fig. 1(a). By means of ENDOR spectroscopy, resolution enhancement can be achieved allowing the observation of distinct signals of two species, one being **1***; the other



Scheme 2. Equilibria of the substrates with BA and **R** and pathways of oxidative radical generation.

Table 1. hf parameters of **1***, **1*** + BA and **1*** + **R** ⇌ **R1***

Parameter	**1*** (232 K)		**1*** + BA (232 K)	**R1*** (203 K)[b]	**R1*** (232 K)[c]	
a_H (mT)	0.684	0.695[a]	0.645	0.515	0.530	0.537[a]
a_N (mT)	0.178	—	0.210	0.139	0.142	—
a_{H_m} (mT)	0.178	0.181[a]	0.186	0.176	0.179	0.181[a]
a_{H_γ} (mT)	0.051	0.049[a]	0.044	0.078	0.071	0.071[a]
Fig.	1(a), (b)				1(a), (b)	

[a] hf constants obtained from ENDOR experiments at $T = 243$ K.
[b] Original ratio: **R**:**1*** = 3:1.
[c] Original ratio: **R**:**1*** = 1:1.

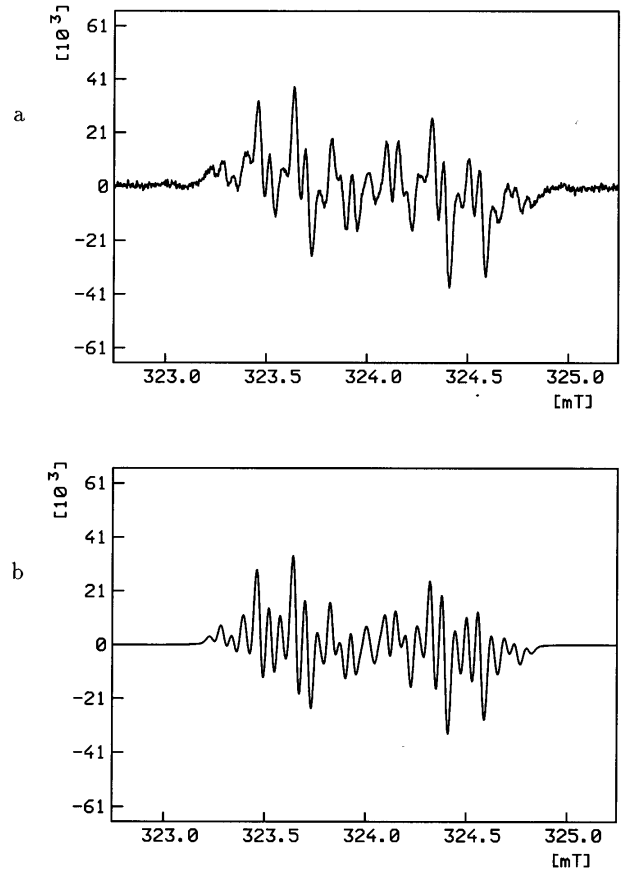


Figure 1. EPR spectrum of the equilibrium **1*** + **R** ⇌ **R1*** at T = 232 K. (a) Experimental spectrum. Original mixture **1***:**R** = 1:1. (b) Simulation. Calculated mixture **1***:**R1*** = 0.55:0.45.

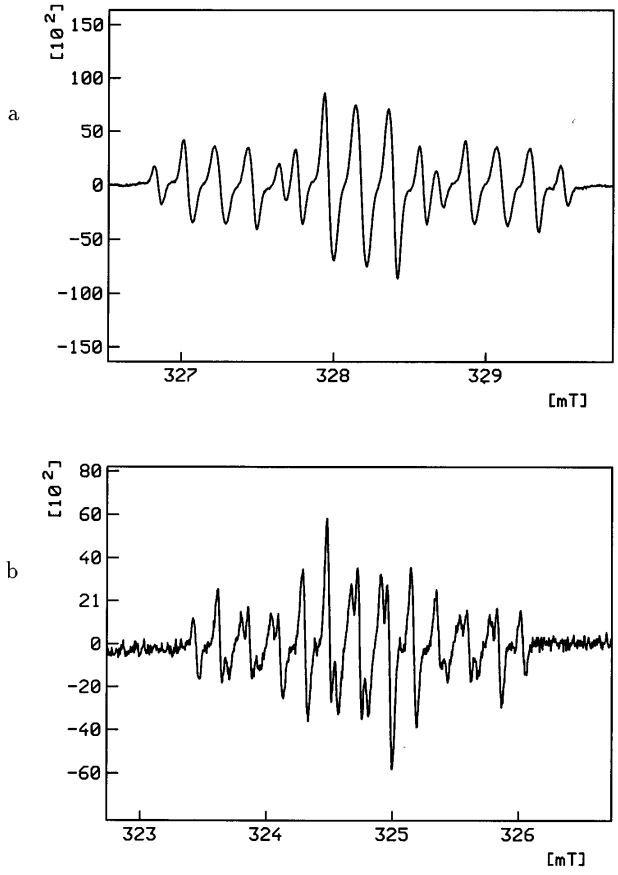


Figure 2. (a) EPR spectrum of free **2*** at T = 293 K. (b) EPR spectrum of **2*** + BA at T = 293 K.

is attributed to **R1*** (for the corresponding ENDOR spectra, see Ref. 1). Superimposing the hyperfine pattern based on coupling constants observed in ENDOR experiments with the hyperfine structure of free **1*** at a **1***:**R1*** ratio of 0.55:0.45 leads to a good agreement with the **1*** + **R** ⇌ **R1*** spectrum in Fig. 1(a). The simulation is shown in Fig. 1(b).

At a receptor:substrate ratio of 3:1 and a temperature of 203 K, the EPR spectrum of **R1*** is characterized by the parameters given in Table 1. No free **1*** is present in the sample, as is also confirmed by ENDOR experiments.

1* + BA. Addition of benzoic acid in the ratio 5:1 to a sample of **1*** leads to a simple spectral alteration: in this case, the spectrum is due to a single paramagnetic species. The value of the nitrogen coupling constant increases compared with free **1*** whereas the β-proton splitting decreases (see Table 1). Both hf parameters decrease on lowering the temperature, a behaviour observed both for the free and the complexed species.

2* and 2* + R

The EPR spectra of the radical **2*** show a hf structure characterized by a large triplet remaining unchanged

Table 2. hf parameters of **2***, **2*** + BA and **2*** + **R** ⇌ **R2***

Parameter	**2*** (293 K)	**2*** + BA (293 K)	**R2*** 252 K	262 K	272 K	282 K	297 K	313 K	323 K	333 K
a_{H_β} (mT)	0.918	0.867	1.090	1.090	1.090	1.090	1.090	1.070	1.070	1.070
a_{H_β} (mT)	0.918	0.867	0.832	0.832	0.832	0.832	0.832	0.832	0.832	0.832
a_N (mT)	0.215	0.238	0.204	0.210	0.217	0.220	0.214	0.223	0.223	0.226
a_{H_m} (mT)	0.188	0.188	0.188	0.188	0.188	0.188	0.194	0.191	0.191	0.191
k (MHz)	—	—	5.5	8.5	17.0	32	55.0	100	170	300
Fig.	2(a)	2(b)	—[a]		—[a]		—[a]			

[a] Published in Ref. 1.

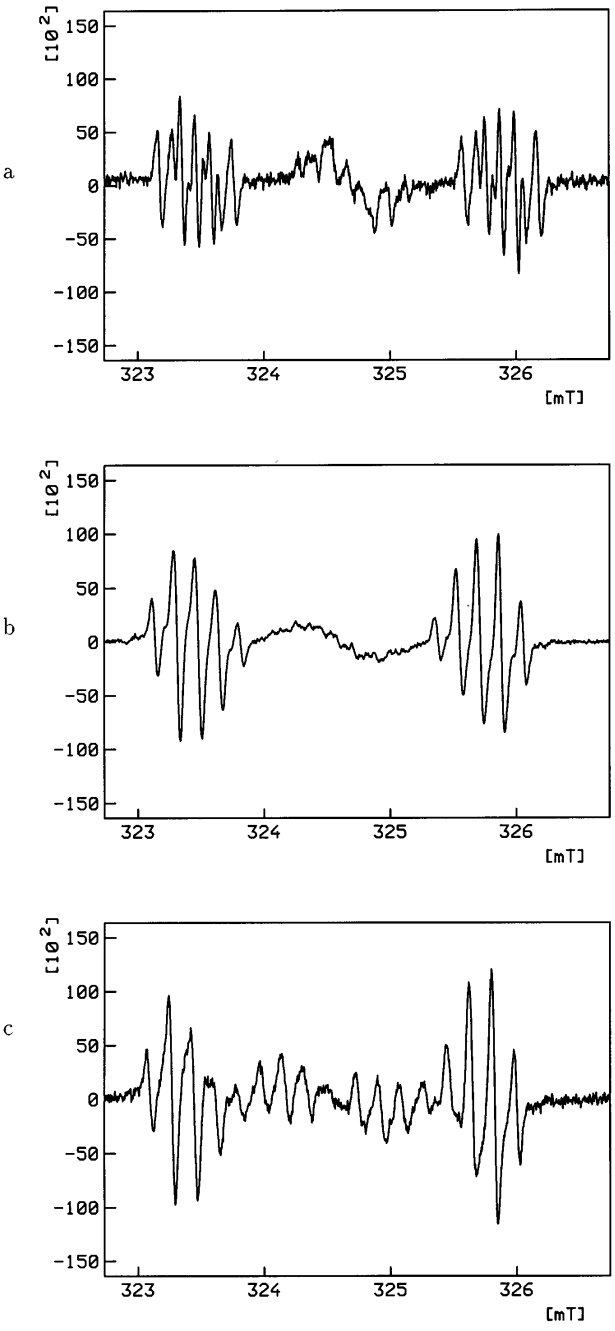


Figure 3. (a) EPR spectrum of free **3*** at T = 232 K. (b) EPR spectrum of **3*** + **R** ⇌ **R3*** at T = 293 K. (c) EPR spectrum of **3*** + **R** ⇌ **R3*** at T = 232 K.

over the experimentally accessible temperature range between 252 and 333 K. The room temperature spectrum is presented in Fig. 2(a). It is easily explained with the coupling constants given in Table 2. However, when **R** is added and the temperature is lowered, the centre lines of the triplet structure undergo strong line broadening. Regaining intensity after coalescence, the hf structure now exhibits a quartet pattern (the series of spectra was published previously[1]). The complete set of hf parameters is given in Table 2. A plot of the exchange frequencies k *vs.* the reciprocal absolute temperature according to Arrhenius yields a straight line leading to an activation energy $E_A = 33.7 \pm 1.1$ kJ mol^{-1} (8.1 ± 0.3 kcal mol^{-1}) and an intercept $\log K_0 = 13.7 \pm 0.2$ (the errors given refer to the linear regression within the plot). Assuming a temperature-independent Δa observed at $T = 252$ K allows the estimation of the exchange frequency at coalescence via the approximation $k_c = \Delta a \times 62.2$ MHz and, thus, of a coalescence temperature $T_c = 271$ K, which is in good agreement with the experimental spectrum (the approximation formula is adopted from NMR for use in EPR spectroscopy, i.e. $[\Delta a] = mT$, and is discussed elsewhere[7]). Consequently, the free activation enthalpy ΔG_c^* can be estimated by means of $\Delta G_c^* = 19.14\ T_c^*\ [10.32 + \log(T_c/k_c)]$ kJ mol^{-1} (Refs 9 and 10) to be 28.8 kJ mol^{-1} (6.9 kcal mol^{-1}).

2* + BA. The presence of acids has a strong influence on the hfs of phenoxyl **2***, as can be seen from Fig. 2(b): the triplet splitting found with **2*** remains dominant whereas the nitrogen coupling is significantly altered (see the data in Table 2). Within the experimentally accessible temperature range, broadening of the central lines could not be observed.

3* and 3* + R

The observation of **3*** over a temperature range of about 100 K reveals that the spectral features change from a large triplet to a broadening of the central lines, illustrated in Fig. 3(a) for 232 K. The hf parameters at 232 and 293 K are given in Table 3. Addition of **R**

Table 3. hf parameters of **3***, **3*** + BA and **3*** + **R** ⇌ **R3***

				R3*			
	3*			232 K		293 K	
Parameter	232	293 K	3* + BA (293 K)	A	B	A	B
a_{H_β} (mT)	1.144	1.144	0.816	1.462	1.259	1.424	1.188
a_{H_β} (mT)	1.273	1.214	1.308	0.700	1.054	0.757	1.116
a_N (mT)	0.116	0.125	0.179	0.178	0.141	0.177	0.140
a_{H_m} (mT)	0.179	0.182	0.179	0.177	0.177	0.180	0.180
Population	—	—	—	0.51	0.49	0.44	0.56
k (MHz)	11.1	50.5	120	5.8	4.8	22.2	17.5
Fig.	3(a)			3(c)	3(c)	3(b)	3(b)

changes the original hf structure of **3*** to that illustrated in Fig. 3(b). When the temperature decreases, the hfs in Fig. 3(b) close to coalescence loses the broadening of the central lines and reveals sharp lines regaining intensity [Fig. 3(c)]. The interpretation can be achieved with the data in Table 3, in good agreement with the experimental spectra.

3* + BA. An excess of benzoic acid (ratio 5:1) affects the hfs of **3*** in a similar way as already discussed for **2***: the addition of BA leads to a strong increase in the nitrogen coupling constant and a decrease in the average value of the β-proton splitting, as indicated by the hf parameters in Table 3. A systematic temperature variation reveals the coalescence point and also the splitting of the two β-protons inequivalent at low temperature.

DISCUSSION

When molecules interact, their spectroscopic properties are known to change. Therefore, whether averaged or distinct signals of the free and the influenced species are observed depends on the spectroscopic time-scale of the method applied. In the course of our investigation, specific lock-and-key interactions proved to be slow within the EPR time-frame, and therefore superimposed spectra of the different species are obtained. On the other hand, no evidence was found for superimposed spectra when non-specific interactions, i.e. mere protonation equilibria, were studied. Nevertheless, both specific and non-specific interactions exhibit a characteristic influence upon the intramolecular dynamic behaviour of the substrates, a phenomenon which is resolved by EPR spectroscopy.

All three substrates reflect a general change of their EPR spectra when BA is added: the nitrogen coupling constant increases, whereas that of the β-proton for **1*** and the average value of the two β-protons for **2*** and **3*** decrease. Assuming that possible protonations at the heterocyclic ring nitrogen atoms would not lead to a significant effect upon the hf components observed, we trace the reported spectral alterations back to protonation of the nitrogen at the methylene group. The same effect can be found with 2,6-di-*tert*-butyl-4-(dimethylaminomethyl)phenoxyl when the solvent is varied systematically: from apolar through polar to protic solvents, the nitrogen coupling constant increases dramatically, while the average value of the β-protons is lowered. The *meta*-protons (H_m) of the phenoxyl remain nearly unchanged [$a_N = 0.188$ mT (toluene), 0.203 mT (acetone), 0.238 mT (ethanol); $\bar{a}_{H_\beta} = 1.098$ mT (toluene), 1.082 mT (acetone), 0.930 mT (ethanol)]. Hence the electronic and steric properties of the nitrogen are altered: while a neutral nitrogen is known to prefer a position in the vicinity of the phenoxyl plane,[4] quaternization directs it towards a perpendicular position leading to a higher coupling constant according to the McConnell–Heller equation.[11] Consequently, the dihedral angle of the β-protons is also altered, lowering the average value of their coupling constants. The electronic influence upon the nitrogen coupling constant cannot be explained as easily.

The EPR spectra of **R1***, **R2*** and **R3*** proved to be more complicated and did not show a similarly uniform behaviour: while both the nitrogen and the β-proton coupling constant are decreased in **R1*** compared with free **1***, a_N remains nearly unchanged in **R2*** but $\bar{a}_H$ decreases. In the complex **R3***, two species are present with a larger nitrogen coupling constant and a smaller average value of the β-proton hf components. Both species show an exchange indicated by the broadening of the central lines.

We attribute this non-uniform behaviour to different orientations of the substrates in the receptor and to different stabilities of the complexes formed. The effect of the intramolecular dynamic flexibilities of **2*** and **3*** within the complex might also be considered to influence the hf values observed. For purine derivatives such as **1*** and **2***, the existence of different orientations within the complex has been discussed:[12,13] the lock's acridine base and the purine moiety are oriented either face-to-face allowing π-stacking interactions or edge-to-face. Hydrogen bonds are likely to be formed between the carboxyclic groups of the cleft **R** and both the imidazole and the pyrimidine heterocyclic units. Our EPR results indicate that no hydrogen bonding exists to the nitrogen atom in position N^6 of the adenine, otherwise its coupling constant would be increased (as is the case when benzoic acid is added). The change in the β-proton hf values can be explained as a consequence of the McConnell–Heller relationship due to the altered orientation of the β-protons to the phenoxyl plane. Within the accessible temperature range, EPR spectroscopy cannot decide whether different possible orientations of the keys in the lock are realized by a dissociation mechanism or a rearrangement within the complex takes place. However, superimposed spectra of the complexed and the free species can be obtained in addition to those which show merely the hfs of the complex, but no evidence is found for an exchange between free and complexed species for **1***, **R1***, **2*** and **R2***.

Neither free nor complexed **1*** shows any intramolecular dynamic behaviour as far as the CHX-N substituent is concerned. Conformational exchange is found in **2*** when complexed. The two β-protons are assumed to exchange by a two-jump mechanism,[14] a model which leads to good agreement between the experimental and calculated spectra. The two-jump model applies also for the interpretation of the exchange observed with free **3*** and **3*** + BA. Concerning **R3***, the exchange observed within the spectra cannot be explained by a two-jump model as the line broadening of the outer quintets is due to a change in the nitrogen splitting [cf Fig. 3(b)]. In addition, at low temperature four different β-proton and two nitrogen coupling constants are present in the spectrum shown in Fig. 3(c). Therefore, the degeneracy of the two-jump mechanism

is removed and the description of the system has to be extended to a two-site model where two sets of hf components related to distinct positions of the nuclei interchange. The two sets of hf components refer to two species with different orientations and binding sites in the receptor–substrate complex related by an equilibrium. Thus, two complexed species, denoted **A** and **B**, exist within the equilibrium **3*** + **R** ⇄ **R3***. In detail, the average value of the β-coupling constants decreases from free **3*** over **B** to **A** in line with the alteration of a_N: **A** has a much larger nitrogen coupling than **B**, which is slightly larger than that of free **3***. By analogy with the observations when BA is added, we suggest that **A** is attached to the receptor by a hydrogen bond to the exocyclic nitrogen atom, whereas **B** is bound via the intracyclic nitrogen atoms of the benzimidazole moiety.

CONCLUSION

Molecular recognition processes have been analysed by EPR spectroscopy. Intermolecular equilibria of **1***, **2***, **3*** and their lock-and-key complexes with **R**, depending on the concentration and temperature of the components, are resolved within the EPR time-scale.

Non-specific protonation can be distinguished from specific receptor–substrate interaction. Whereas non-specific interaction exhibits a uniform influence on the substrates, specific recognition does not have a generalized effect on the molecules studied. This is interpreted in terms of different stereochemistry and rigidity of **R1***, **R2*** and **R3***. The complexation also changes the intramolecular flexibility of the substrates. Three models, a pure static, a two-jump and a two-site mechanism, were applied to the interpretation of the EPR spectra observed. They are referred to the molecular level, providing information on the intermolecular interactions and the resulting stereochemistry of the species.

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