The Role of Metal Ions in Processes of Conformational Selection during Ligand-Macromolecule Interactions

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1 Introduction

The interaction with macromolecules plays a major role in eliciting the biochemical activity of relatively small flexible ligands. Several processes are involved in such interaction such that the key and hole assumption may often fail. One of the processes not carefully considered so far is that of conformational selection, in which the macromolecule stabilizes the conformation of the ligand at the bound site after a selection among several conformational arrangements. The conformation at the bound state may or may be not connected to some low-energy conformation or to the conformation stabilized at the solid state. The comprehension of this process is expected to provide a valuable aid in rationale drug design where synthesized molecules are sought yielding the same or even more specific responses than the natural ligands.

Investigation of this pro-

cess requires to delineate the change in conformation when going from the free to the bound state and, from this point of view, NMR is the technique of choice.

Here we present evidence that NMR allows to detect and delineate the change in conformation experienced by a flexible ligand, the dipeptide carnosine (β-alanyl-L-histidine), when it binds to the serum protein albumin. We show also that the process of conformational selection is favoured by the presence of divalent metal ions, such as Ca(II) and Cu(II), that stabilize, in the metal complex, a conformation of the ligand very close to that assumed in the bound state.

2 NMR Parameters

The preferred conformation assumed by the ligand in its free state in solution can be easily delineated by measuring dipolar interaction energies between pairs of homo- or hetero-nuclear spin. Since such interaction terms are functions of τ_c/r^6, distances can be calculated if the reorientational dynamics can be characterized, even in some approximate way. This last purpose can be accomplished by measuring and interpreting the ^{13}C-NMR spin-lattice relaxation rates, that are, in general, determined by the one bond (r = 1.09 Å [1]) ^{13}C-^1H dipole-dipole interaction.

Once the motional correlation time(s) is (are) determined, relevant geometric features can be obtained by one or more of the following experiments:

a) evaluation of the ^1H-{^1H} n.O.e. if spectral resolution is not limited and cross-correlation effects can be neglected;
b) measurement of single- and double-selective ^1H-NMR spin-lattice relaxation rates of selected proton pairs; these provide a means of calculating absolute values of pairwise dipolar cross-relaxation terms, σ_{ij} [2-4];
c) measurement of the ^{13}C-{^1H} n.O.e. upon selective presaturation of resolved proton resonances [5];
d) evaluation of relative values of cross-relaxation terms from intensities of cross peaks in 2D NOESY maps [6].

All these methods are very efficient in providing the desired information on the preferred conformation in solution of any 'NMR visible' ligand and, eventually, the change in conformation caused by the presence of metal ions. In this last case, if the metal is paramagnetic, a great piece of structural information is gained by investigating the paramagnetic effects on nu-

clear relaxation rates and chemical shifts [7,8] or on 2D spectra [9,10].

In order to investigate the eventual change in the conformation of the ligand when it binds to a macromolecule either in the presence or in the absence of metal ions, the previously outlined NMR methods, at least not all of them, are not as efficient as in the free state. The concentration of the macromolecule is a limiting factor, since it must be kept quite small if spectral distortion is to be avoided. As a consequence exchange of the ligand between the free (bulk) and the bound state must be taken into consideration, yielding:

$$P_{obs} = x_f P_f + x_b P_b$$

where P is any observed NMR parameter, f and b refer to the free and bound states and the x's are molar fractions. It follows that the change in P from the free to the bound state is expressed by:

$$\Delta P = x_b P_b$$

where x_b is usually of the order 0.01-0.1. The consequence is that spin-lattice relaxation rates and chemical shifts are no longer suitable parameters for delineation of geometric features of the bound state, unless a paramagnetic centre, either intrinsic or extrinsic, is present. One is then left with measurements of 1D or 2D transferred n.O.e. or, which we prefer, of single- and double-selective proton spin-lattice relaxation rates. In fact, in absence of spectral distortions, the same measurements can be easily accomplished for the free ligand as well as in the system where the ligand is exchanging between the two states, and absolute values of the cross-relaxation rate can be separately obtained for the free and the bound ligand.

3 Free Carnosine

The relevant features of carnosine in water solution can be summarized as follows:

a) reorientational dynamics can be interpreted in terms of a principal correlation time (τ_c = 58 ps) describing reorientation around a molecular axis passing through the imidazole ring, coupled with segmental motion of the amino-terminal moiety and librational motion of the ring (τ_g = 10 ps);

b) predominance of the g⁻ rotamer around the C_6-C_7 bond (CH_2-CH segment of the histidyl residue);

c) folding of the β-alanyl moiety towards the imidazole ring.

4 Calcium Complex

Calcium forms two complex speies with carnosine in solution: a 1:1 complex where the carbonyl and carboxyl oxygens are the metal binding atoms and a dimeric complex where the two carbonyl and one carboxyl oxygens and the imidazole nitrogen are the four coordinated atoms. The overall dissociaton constant of the complexes is K_d = 0.04 mol dm^{-3}. In the monomer complex the dipeptide retains the conformation detected by NMR as the 'preferred' one in the free state in solution. In the dimer species extensive intermolecular interactions are favoured and the conformation of the dipeptide is less folded than in the free state or in the 1:1 complex.

It is concluded that calcium ions stabilize a particular geometric arrangement that is itself representing the 'preferred' conformation in solution, as it raises from motional averaging of all the particular conformations assumed by the flexible peptide.

5 Interaction with HSA

The interaction of carnosine with human serum albumin (HSA) can be detected and delineated by measuring selective and double-selective proton spin-lattice relaxation rates of carnosine protons in the presence of low molar fractions of the protein [11,12]. Detection of binding is allowed by appreciable relaxation rate enhancements of selective relaxation rates of the imidazole protons, as well as of the His $H\alpha$:

$$\Delta R^{sel} = p_b R_b^{sel}$$

Even at very low fractions of bound carnosine the selective relaxation rate at the bound site is so fast that the observed rate undergoes enhancements as high as 50-100 %. The effect allows also a titration of the binding process, yielding an apparent dissociation constant of 2.5×10^{-4} mol dm^{-3}.

The observed enhancements are consistent not only with a very tight binding of the whole peptide molecule to the protein but also with occurrence of dipolar interaction between ligand and protein protons, although there is no possibility of obtaining quantitative estimations of such interactions.

More information can be obtained by measuring the double-selective proton spin-lattice relaxation rates within the His $H\alpha$-$H\beta_1$-$H\beta_2$ moiety. As in the free state in solution, such measurements yield the dipolar cross-relaxation rate between the excited protons, e.g.:

$$\sigma\alpha,\beta1 = R_{\alpha}^{\alpha,\beta1} - R_{\alpha}^{sel}$$

where the first term on the right hand defines the double-selective spin-lattice relaxation rate measured on $H\alpha$ when both $H\alpha$ and $H\beta1$ are excited.

The cross-relaxation rate in the bound ligand is obtained by:

$$\sigma b = \frac{\sigma obs - \sigma f}{pb}$$

As a consequence of binding, the cross-relaxation rate changes from positive to negative values and allows to gain geometric information on the bound molecule.

In fact, substitution of the motional correlation time of the protein in the equation:

$$\sigma b = -0.1 \frac{\gamma^4 h^2}{r^6} \tau c$$

provides a means of evaluating proton-proton distances in the bound peptide. It comes out that at least the investigated moiety retains the conformation that was shown to be stabilized by calcium ions with exclusive occurrence of the g⁻ rotamer.

6 Effect of Calcium

The same experiments used to detect and delineate binding of carnosine to HSA can be repeated in the presence of Ca(II) at equimolar ratios with the ligand. No substantial change is observed, as far as the selective relaxation rate enhancement and the change in the double-selective relaxation rates are concerned. An appreciable change can be however observed in the ligand-protein dissociation constant that is now measured at 2.0×10^{-5} *mol* dm^{-3}.

It is therefore possible to conclude that the effect of the metal ion is to stabilize the conformation that occurs at the bound site. In absence of the ion, such conformation has to be selected among all the several conformations that are possibly assumed by the flexible ligand in solution. This process leads to an appreciable reduction of the binding constant.

7 Effect of Copper

It is important to underline that the same experiments cannot be carried on when using a paramagnetic ion such as copper. It is still possible to delineate the geometry of the metal complex, but, only of that having the maximum number of ligands in the coordination sphere. One is in fact forced to work at very low [metal]/[ligand] ratios.

By the same way, it is possible to detect and delineate the ternary complexes

formed in the presence of the protein but there is no way of shedding light on the geometrical and conformational features of the ligand bound to the macromolecule.

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