ANION BINDING TO PROTEINS. NMR QUADRUPOLE RELAXATION STUDY OF CHLORIDE BINDING TO VARIOUS HUMAN HEMOGLOBINS

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1. Introduction

Two n.m.r. spectroscopic approaches to the study of protein binding of anions can be distinguished. In the first binding is monitored by observing the changes in the positions and/or linewidths of the signals in the ^{1}H or ^{13}C n.m.r. spectra as a result of anion binding. For example when the anion is paramagnetic like $Cr(CN)_6^{3-}$ the site of binding to lysozyme, carbonic anhydrase, and cytochrome *c* have been inferred from the ^{1}H n.m.r. spectra [1–4].

The second approach is based upon the fact that many simple and complex anions have nuclei with magnetic moments and are more or less suitable for direct n.m.r. studies. Binding influences the relaxation rate of the nuclei of the bound ions. Under conditions of fast exchange the interaction with the protein can be conveniently followed by studying the n.m.r. signal from the anion in the bulk solution. The interpretation of the relaxation changes is particularly simple for magnetic nuclei with electric quadrupole moments, such as chloride and bromide [5]. This type of technique has been employed for the study of chloride binding to human hemoglobin. The picture which has emerged so far is that high affinity binding occurs at specific regions of the protein [6,7]. In these studies the experiments performed were of two kinds: (i) competition experiments with other anions with known binding sites, notably organic phosphates, and (ii) comparison of the chloride binding pattern of Hb A with those of mutant or chemically modified hemoglobins. It appears that chloride is bound with high affinity not only at the organic phosphate binding region (involving the N-terminal of the beta chains, Hisβ-2, Lysβ-82 and Hisβ-143), but also at (or near) the C-terminal residues of both types of chains (Hisβ-146–Aspβ-94 and Valα-1–Argα-141). Direct evidence has also been obtained for the linkage of the binding of chloride with that of oxygen and protons [7].

This note reports on the chloride binding properties of various human hemoglobins with structural modifications in the proposed chloride binding regions: Hb A_{IC}, where the N-terminal of the beta chains is blocked by a Schiff base, Hb F, where the position corresponding to Hisβ-143 is occupied by a serine, and Hb F_1, where in addition the N-terminal of both gamma chains is acetylated. The results obtained confirm the previous assignments of the high affinity binding sites and thus substantiate the idea that the chloride binding properties of HbA result from high affinity binding to a few specific, localized binding sites.

2. Materials and methods

The hemoglobins A, A_{IC}, F and F_1 were prepared as described by Mansouri and Winterhalter [8].

The n.m.r. measurements were performed as described by Chiancone et al. [6,7]. The hemoglobin samples were equilibrated with the appropriate NaCl solutions, containing 10^{-4} M EDTA, by passage through a Sephadex G-25 column.

3. Results and discussion

The binding of Cl^- to hemoglobins HbA_{IC}, HbF and HbF_1 could be conveniently followed through measurements of the ^{35}Cl n.m.r. linewidth on a few tenths molar sodium chloride solution in the presence of small amounts of the protein. Since chemical exchange is rapid, binding results in an increase in linewidth in excess of that of the free ion. The excess broadening depends on the number (n_i) of ions bound and on the linewidth ($\Delta\nu_i$) characteristic of each binding site (for a recent review see [5]).

The strong dependence of the linewidth on chloride concentration observed for all hemoglobins indicates the presence of high affinity binding sites like in the case of HbA. The decrease in linewidth upon deoxygenation is also similar to that observed for HbA and is similarly indicative of a stronger interaction of the ion with the deoxygenated derivative (table 1). The latter results, implying the presence of a chloride–oxygen linkage, parallel the effect of organic phosphates on the oxygen equilibrium [8,9].

The effect of pH was analysed in view of the information it may provide on the nature of the chloride binding sites. Fig.1 shows the results obtained on 1% solutions of HbA_{IC} in 0.5 M NaCl. The excess

Table 1
Dependence of the ^{35}Cl excess linewidth on NaCl concentration and on the presence of a heme ligand for HbA_{IC}, HbF and HbF_1

	NaCl conc. (M)	Derivative	$\Delta\nu_e$ (1%)
HbA_{IC}	0.50	oxy	6.5
	0.50	CO	7.0
	0.50	deoxy	3.8
	0.35	oxy	7.2
	0.35	deoxy	6.0
	0.30	CO	10.2
	0.30	deoxy	6.6
	0.2	oxy	13.9
	0.2	deoxy	6.2
HbF	0.50	oxy	13.4
	0.50	deoxy	5.2
	0.30	oxy	19.5
	0.20	oxy	22.2
	0.20	deoxy	6.8
HbF_1	0.2	oxy	18.1
	0.2	deoxy	7.5

Protein concentration 1%; pH 6.8–7.3.

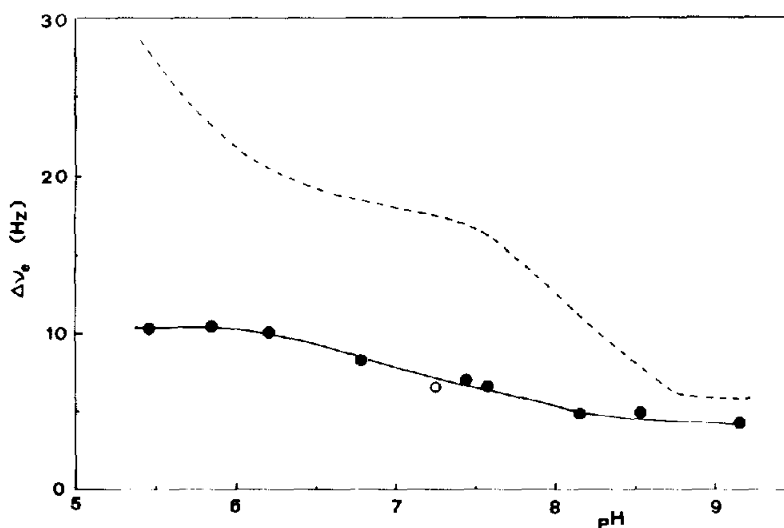

Fig.1. Excess linewidth as a function of pH in the presence of HbA_{1C}. Solvent: 0.5 M NaCl, hemoglobin concentration 1%, hemoglobin derivative: (○) oxy, (●) carbonmonoxy. Dashed line refers to the CO derivative of HbA under the same experimental conditions (from [6]).

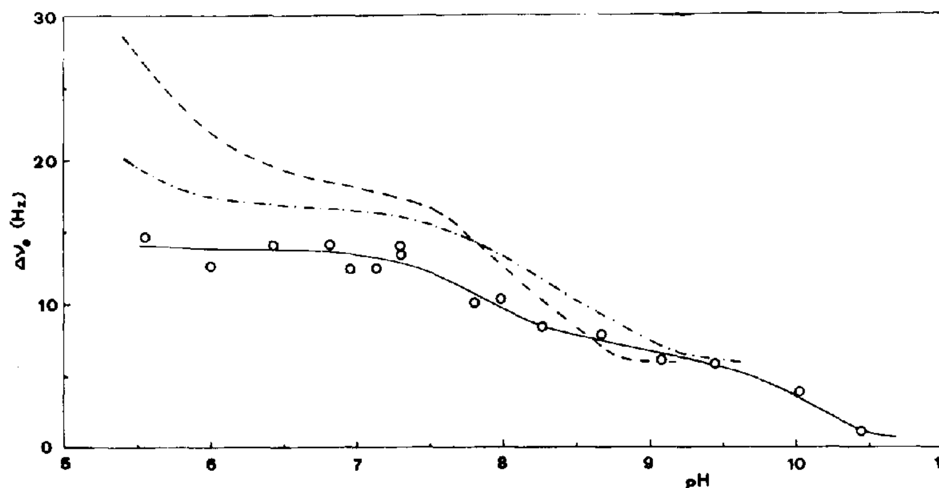

Fig.2. Excess linewidth as a function of pH in the presence of HbF. Solvent: 0.5 M NaCl, hemoglobin concentration 1%, hemoglobin derivative: oxy. Dashed lines refer to the CO derivatives of HbA (– – – –) and Hb Abruzzo (-. - . - . -) respectively (from [11]).

linewidth observed in the solutions of HbA_{IC} is markedly reduced at all pH values in comparison with HbA. In addition the pH profile of HbA_{IC} lacks the inflection point at slightly alkaline pH values. In HbA this has been attributed to the neutralization of amino groups; in particular, given the competition between chloride and organic phosphates, the N-terminal groups of the beta chains appeared as good candidates also for chloride binding. Thus the significant reduction in linewidth in this pH region can be explained as resulting directly from the blocking of the N-terminal residues of the beta chains. The flatness of the excess linewidth at acid pH values can be accounted for on the same basis. In HbA the strong pH dependence of the linewidth in this pH region has been ascribed to amino groups which are rendered accessible for chloride binding by the progressive neutralization of neighboring carboxyl groups [7]. In HbA_{IC} the blocking of the N-terminal amino groups simply prevents chloride binding in this region; therefore the neutralization of neighboring carboxyl groups should have no effect on the chloride linewidth.

Fig.2 shows the pH dependence of the excess linewidth of a 1% solution of HbF in 0.5 M NaCl. With respect to HbA the linewidth is somewhat lower and is characterized by the lack of the inflection point at slightly acid pH values and by a second inflection point at about pH 10. Both these features can be interpreted on the basis of the structure of HbF and HbA. The titration point at about pH 10 can be attributed to the ionization of the ε-amino group of a lysine residue; the most likely one being that of Lysγ-82, which is involved in organic phosphate binding. In HbA, which is not alkali resistant, this titration point could not be reached. The constancy of the linewidth in the acid region can be due to the substitution of Hisβ-143 with a serine, a neutral amino acid. The close similarity between the pH profile of HbF and that of Hb

Table 2
Dependence of the ^{35}Cl excess linewidth on pH for HbF_I and HbF.

	Derivative	pH	$\Delta\nu_e$ (1%)
HbF_I	oxy	6.80	18.1
	CO	7.39	18.4
	CO	8.04	7.7
HbF^a	oxy	6.80	22.6
	oxy	7.39	20.8
	oxy	8.04	16.0

Protein concentration 1% in 0.2 M NaCl
[a]Data recalculated from table 1 and Fig.2.

Abbruzzo (Hisβ-143 → Aspβ-143) (cf. fig.2) strengthens this interpretation [11].

The results obtained with HbF_1 are somewhat limited in view of the difficulty of obtaining big quantities of the material, which required linewidth measurements at a very low chloride concentration (0.2 M). Assuming the same type of dependence on NaCl concentration as for HbF, then HbF_1 has a lower linewidth at all pH values (cf. table 2). This finding is consistent with the blocking of the N-terminal residues of the gamma chains.

In conclusion the results obtained confirm a common chloride binding pattern arising from the contribution of a few well localized high affinity binding sites; of these some also bind organic phosphates.

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