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NUCLEAR MAGNETIC RESONANCE QUADRUPOLE RELAXATION STUDIES OF CHLORIDE BINDING TO THE ISOLATED HEMOGLOBINS FROM TROUT (Salmo irideus)

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NMR studies of chloride binding to the main components of trout blood, Hb Trout I and Hb Trout IV, indicate that although the affinity of chloride is high for both hemoglobins, the characteristics of the binding process are markedly different. In Hb Trout IV chemical exchange at the chloride binding site(s) is fast and quadrupole effects determine the linewidth; chloride binding has a definite pH dependence, but there is no significant oxygen linkage. In contrast Hb Trout I represents a unique case of slow chemical exchange, which may depend on unusual stereochemical characteristics of the chloride binding site; chloride binding is pH independent, but shows a significant oxygen linkage, which may be attributed to changes of the lifetime of chloride at the binding site.

The chloride binding properties displayed by Hb Trout I and IV have been compared with those of normal and modified human hemoglobins and discussed in terms of the structural differences in the C- and N-terminal regions of the α- and β-chains.

1. Introduction

The hemolysate of trout's blood contains four main electrophoretically distinct components. The two major ones, designated as Hb Trout I and Hb Trout IV, were shown to have strikingly different functional properties [1, 2]: the Root effect – the pH dependent change in cooperativity and oxygen affinity characteristic of fish hemoglobins – is observed only in Hb Trout IV. In contrast the oxygen affinity of Hb Trout I is invariant with pH, although the equilibrium curve is markedly cooperative. The effect of the physiologically important organic phosphate, ATP, is also different for the two major hemoglobins from trout: the functional properties of Hb Trout I are unaffected, while those of Hb Trout IV are largely changed [3]. Inorganic salts, like sodium chloride, have a small, but measurable effect on the oxygen affinity of both components (to be published).

Nuclear magnetic resonance quadrupole relaxation studies have been successfully employed for the characterization of the interaction of chloride ions with normal and chemically modified human hemoglobins [4, 5]. In particular the studies of the chemically modified hemoglobins have allowed a tentative identification of chloride binding sites, only some of which overlap with the organic phosphate binding site. In the present paper similar studies of chloride binding by the two major hemoglobin components from trout will be presented. Hb Trout IV is shown to exhibit similarities, as well as differences with respect to human hemoglobin. In contrast, Hb Trout I represents a unique case in so far as chemical exchange dominates the chloride relaxation.

2. Materials and methods

Hemoglobin from trout "Salmo irideus" was prepared and separation of components was achieved as described by Binotti et al. [2]. Concentrations were determined on the basis of $E^{1\,cm}_{1\%} = 8.4$ at 540 nm for the carbonmonoxide derivative. Samples for the NMR measurements were prepared by mixing appropriate aliquots of a deionized hemoglobin stock solution with 5M NaCl (the commercial Suprapur Merck pro-

duct). In order to minimize autoxidation of the protein the solutions were usually made 10^{-4}M in EDTA; at the end of the NMR measurements the optical spectra indicated formation of variable amounts of methemoglobin (up to ~20%).

The nuclear magnetic resonance spectra of ^{35}Cl were performed as described previously (Chiancone et al. [4, 5]).

A Hitachi Perkin–Elmer Model 124 double beam recording spectrophotometer was used for the optical density measurements.

The pH was measured on a Radiometer 25 pH meter. In the experiments on the effect of pH on the ^{35}Cl linewidth the pH was measured directly in a Thunberg tube with fitted electrode so designed that it could be introduced into the nuclear magnetic resonance probe.

3. Results

3.1. Dependence of linewidth on NaCl concentration

The dependence of the ^{35}Cl excess linewidth on NaCl concentration at pH near neutrality for the carbonmonoxide derivative of Hb Trout I and Hb Trout IV is shown in fig. 1a and b. The results on Hb Trout I refer to 0.5% solutions, those on Hb Trout IV to 1% solutions.

In view of the variability of the ^{35}Cl linewidth shown by different preparations, measurements on the effect of deoxygenation were of a differential kind. The ^{35}Cl linewidth in the presence of deoxyhemoglobin was measured first in a Thunberg tube, then CO was added and the linewidth measured again. In Hb Trout IV, due to the Root effect, binding of CO results in a decrease in pH (e.g., 0.3–0.4 pH units at pH 6.5–6.7). After correction for the pH change (see below) no significant difference was observed between the deoxy and the CO derivative in the range 0.3–0.8 M NaCl. On the other hand in Hb Trout I a significant decrease in linewidth (20–35%) was found at various chloride concentrations going from deoxy to CO hemoglobin (fig. 1a).

3.2. Dependence of linewidth on pH

The effect of pH on the ^{35}Cl excess linewidth is given in fig. 2a and b for the liganded and unliganded derivatives of Hb Trout I and Hb Trout IV at concentrations of 0.5 and 1% respectively. The data were obtained in 0.5 M NaCl solutions in which the pH was adjusted by addition of small amounts of 0.05–0.3 M NaOH and HCl.

3.3. Linewidth in the presence of various reagents

The effect of various reagents on the ^{35}Cl excess linewidth in the presence of the carbonmonoxide derivative of Hb Trout I and Hb Trout IV is given in table 1. The reagents analyzed include EDTA, 2,3–dimercaptopropanol (BAL), inositolhexaphosphate (IHP) and gold and platinum cyanates (K Au $(CN)_2$, $K_2Pt(CN)_4$). The latter have been shown to compete with Cl ions for aminoacid sidechains [6].

3.4. Dependence of linewidth on temperature

The dependence of the ^{35}Cl excess linewidth on temperature for the liganded and unliganded derivatives of Hb Trout I and Hb Trout IV is shown in fig. 3,

Table 1
Effect of various reagents on the ^{35}Cl linewidth in the presence of the carbonmonoxide derivative of Hb Trout I and Hb Trout IV

Component	Addition	$\Delta\nu_{exc}$ (Hz)
Hb Trout I	0.5 M NaCl	23.1
(0.5%; pH 6.5)	0.5 + 6×10^{-4} M EDTA	24.2
	0.5 + 6×10^{-2} M BAL	22.0
	0.5 + 5×10^{-5} M K $Au(CN)_2$	20.1
Hb Trout I	0.35 M NaCl	30.9
(0.5%; pH 5.9)	0.35 + 2.4×10^{-3} M IHP	23.2
Hb Trout I	0.35 M NaCl	38.2
(0.6%; pH 6.0)	0.35 + 3.5×10^{-5} M IHP	33.6
	0.35 + 7.0×10^{-5} M IHP	34.2
	0.35 + 1.0×10^{-4} M IHP	32.8
	0.35 + 1.4×10^{-3} M IHP	31.6
Hb Trout I	0.35 M NaCl	34.5
(0.6%; pH 6.5)	0.35 + 3×10^{-3} M K $Au(CN)_2$	34.3
	0.35 + 9×10^{-3} M K $Au(CN)_2$	32.9
	0.35 + 1×10^{-3} M $K_2Pt(CN)_4$	30.1
Hb Trout IV	0.35 M NaCl	14.1
(1.3%; pH 6.5)	0.35 + 2×10^{-4} M IHP	12.3
	0.35 + 1×10^{-3} M IHP	12.4

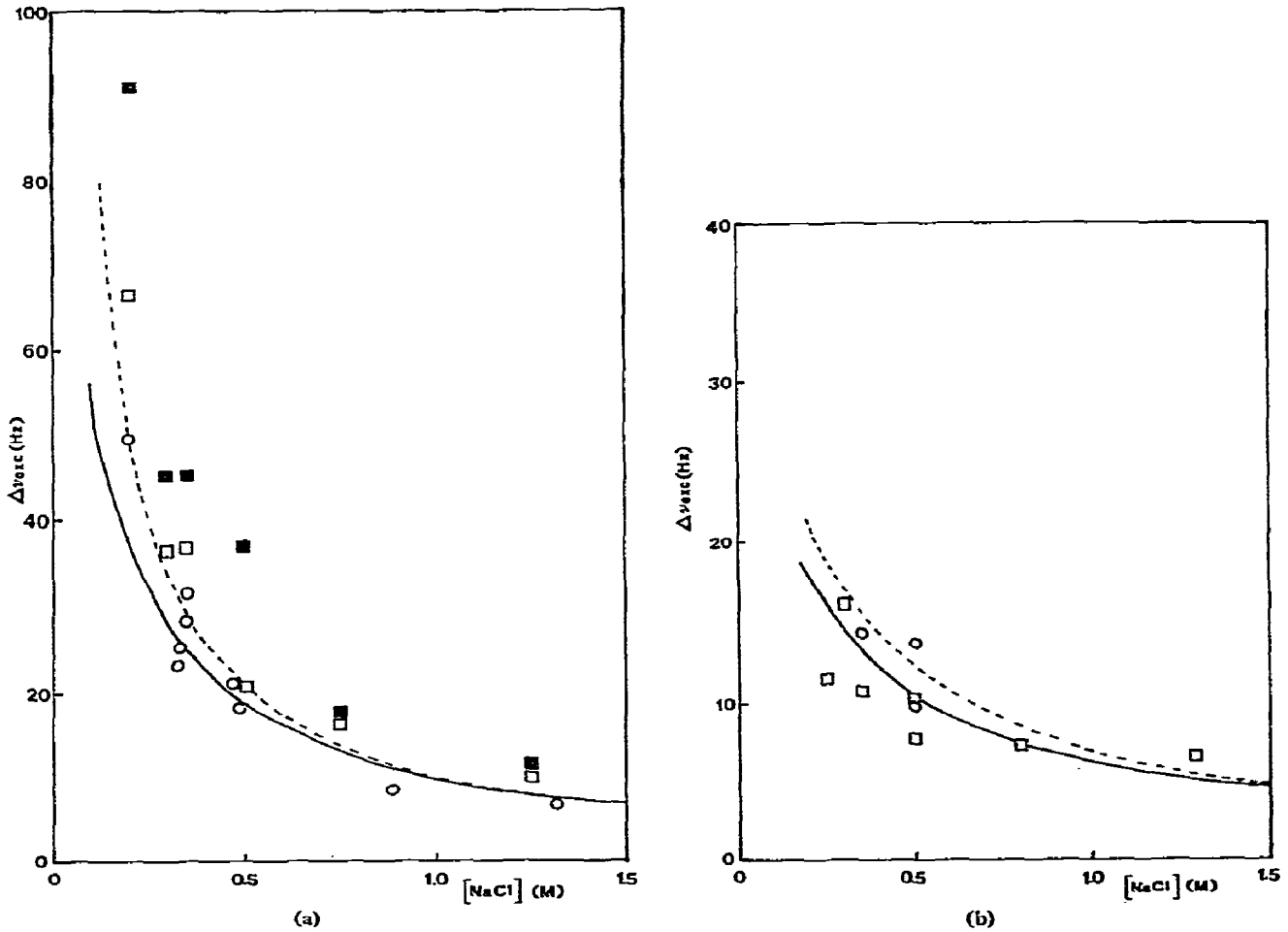

Fig. 1. Excess linewidth as a function of NaCl concentration in the presence of Hb Trout I (a) and Hb Trout IV (b) at pH 6.5–6.8. Protein concentration: 0.5% (a), 1% (b). Different symbols refer to different preparations. (○, □) carbonmonoxide; (■) deoxygenated derivatives. Lines are theoretical ones and were calculated with the following binding constants: for HbCO Trout I (—) $K_A = 10\ M^{-1}$, (----) $K_A = 100\ M^{-1}$; for HbCO Trout IV (—) $K_A = 10\ M^{-1}$, $K_B = 0.1\ M^{-1}$; (----) $K_A = 5\ M^{-1}$.

where the logarithm of linewidth is plotted as a function of $1/T$, T being the absolute temperature. The data for Hb Trout I were obtained on 0.5% solutions in 0.5 M NaCl, those for Hb Trout IV were obtained on 1.6% solutions in 0.35 M NaCl at pH 6.5–6.7.

3.5. Dependence of linewidth on frequency

The frequency dependence of the ^{35}Cl excess linewidth in a 0.5 M solution of NaCl containing 1.45% carbonmonoxide Hb Trout IV at pH 6.7 is shown in fig. 4.

3.6. Linewidth for the two chloride isotopes (^{37}Cl and ^{35}Cl)

The linewidth measurements for the two isotopes ^{37}Cl and ^{35}Cl were performed at the same frequency, namely 4.88 MHz, using magnetic fields of 14.04 and 11.69 kG respectively. The results for both hemoglobin components, Hb Trout I and Hb Trout IV in 0.5 M NaCl are given in table 2.

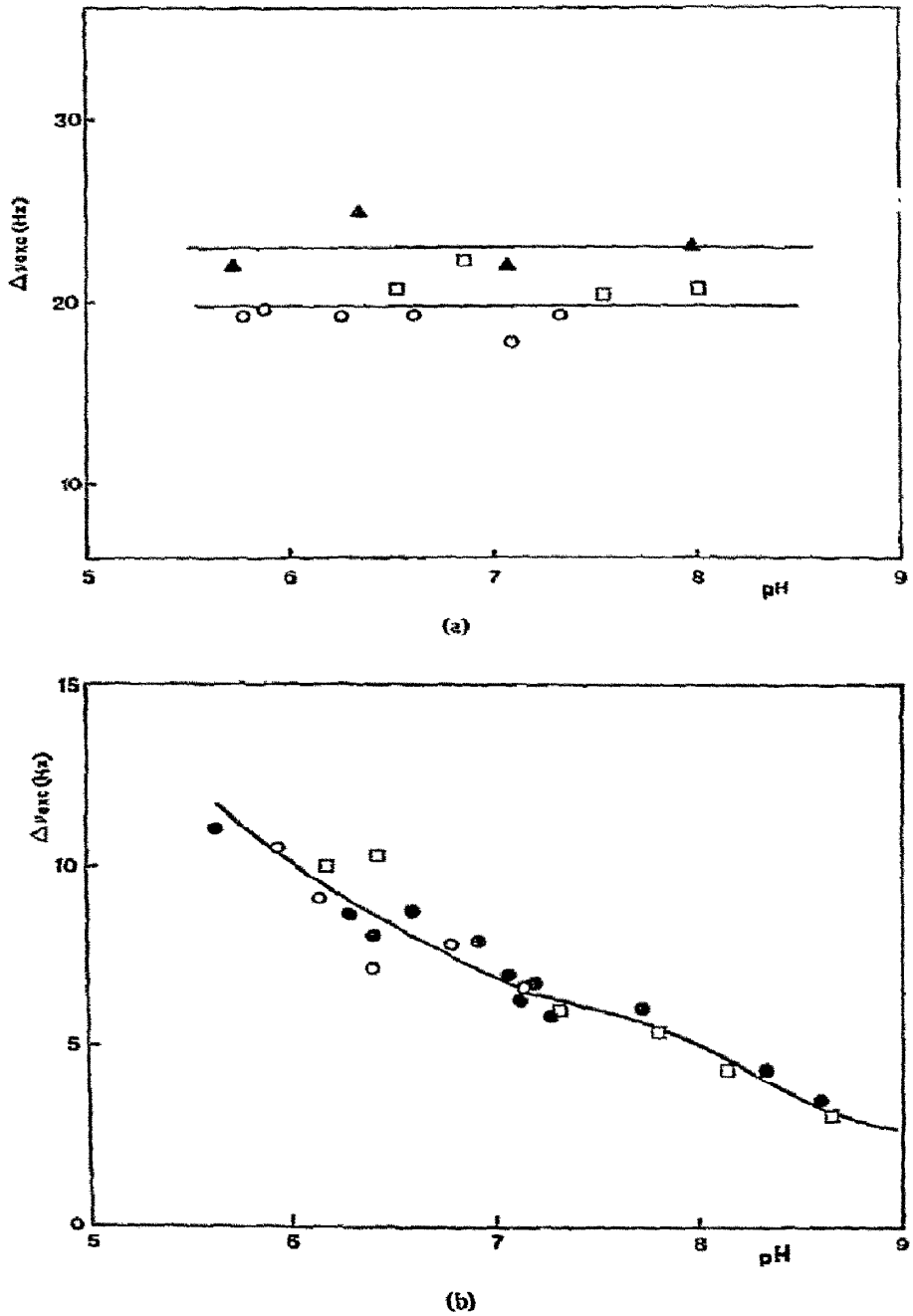

Fig. 2. Excess linewidth as a function of pH in the presence of Hb Trout I (a) and Hb Trout IV (b). Solvent: 0.5 M NaCl. Protein concentration: 0.5% (a), 1% (b). Hemoglobin derivative: (○, □) HbCO, (▲, ●) Hb. Different symbols refer to different preparations.

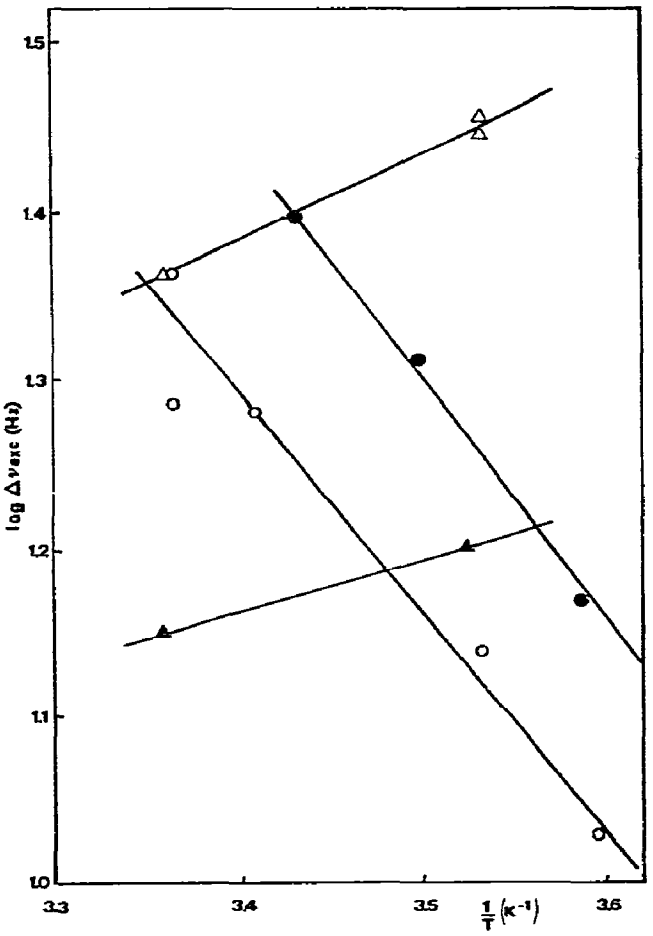

Fig. 3. Excess linewidth as a function of absolute temperature in the presence of Hb Trout I and Hb Trout IV at pH 6.5–6.8. Solvent: 0.5 M NaCl for Hb Trout IV, at 1.6%, (△, ▲) and 0.5 M NaCl for Hb Trout I, at 0.5%, (○, ●). Hemoglobin derivative: (○, △) HbCO, (●, ▲) Hb.

4. Discussion

For ions of magnetic nuclei with electric quadrupole moments like $^{35}Cl^-$ and $^{37}Cl^-$, which undergo chemical exchange in solution with different sites on a protein at sufficient speed to give only one condensed signal, the observed linewidth, $\Delta\nu_{obs}$, may be written as

$$\Delta\nu_{obs} = f_0\Delta\nu_0 + \sum_i f_i\Delta\nu_i, \qquad (1)$$

where f_0 represents the fraction of free ions in solution with the characteristic linewidth $\Delta\nu_0$ and f_i is the fraction bound to site i on the protein with the characteristic linewidth $\Delta\nu_i$. Since in our experiments the chloride ion concentration was always at least 10^4 times the protein concentration we can put $f_0 \approx 1$ and define an "excess" linewidth, $\Delta\nu_{exc}$, for the chloride NMR signal as

$$\Delta\nu_{exc} = \Delta\nu_{obs} - \Delta\nu_0 = \sum_i f_i\Delta\nu_i.$$

From mass law considerations we may write [4]

$$f_i = n_i[P]_t K_i/(1 + K_i[Cl^-]_t),$$

where n_i is the number of chloride ions bound at site i with the binding constant K_i, $[P]_t$ equals the total protein concentration and $[Cl^-]_t$ equals the total concentration of chloride.

If one neglects chemical shift differences between free and bound chloride $\Delta\nu_i$ is given by [11]

$$\Delta\nu_i = \frac{1}{\pi T_2^i} = \frac{1}{\pi}\left(\frac{0.6}{T_{2M}^{i\prime} + \tau_M^i} + \frac{0.4}{T_{2M}^{i\prime\prime} + \tau_M^i}\right), \qquad (4)$$

where τ_M is the mean lifetime of the nucleus at site i and

$$\frac{1}{T_{2M}^{i\prime}} = \frac{1}{20}\left(\frac{e^2qQ}{\hbar}\right)^2\left(\tau_c + \frac{\tau_c}{1+\omega^2\tau_c^2}\right), \qquad (4a)$$

and

$$\frac{1}{T_{2M}^{i\prime\prime}} = \frac{1}{20}\left(\frac{e^2qQ}{\hbar}\right)^2\left(\frac{\tau_c}{1+\omega^2\tau_c^2} + \frac{\tau_c}{1+4\omega^2\tau_i^2}\right), \qquad (4b)$$

where $(e^2qQ/\hbar)$ is the quadrupole coupling constant for the chloride nuclei and τ_c the correlation time characterizing the reorientation of the electric field gradient affecting the relaxing nuclei. ω is the resonance frequency in radians per second. Physically, eq. (4) means that when $\tau_M \gg T_{2M}', T_{2M}''$, the life time of the chloride ions at the binding site(s) determines the overall chloride relaxation in solution, $1/T_2$, and hence the linewidth, $\Delta\nu$. On the other hand when $\tau_M \ll T_{2M}', T_{2M}''$, i.e., rapid chemical exchange, the quadrupole relaxation rate at the binding site will determine the linewidth.

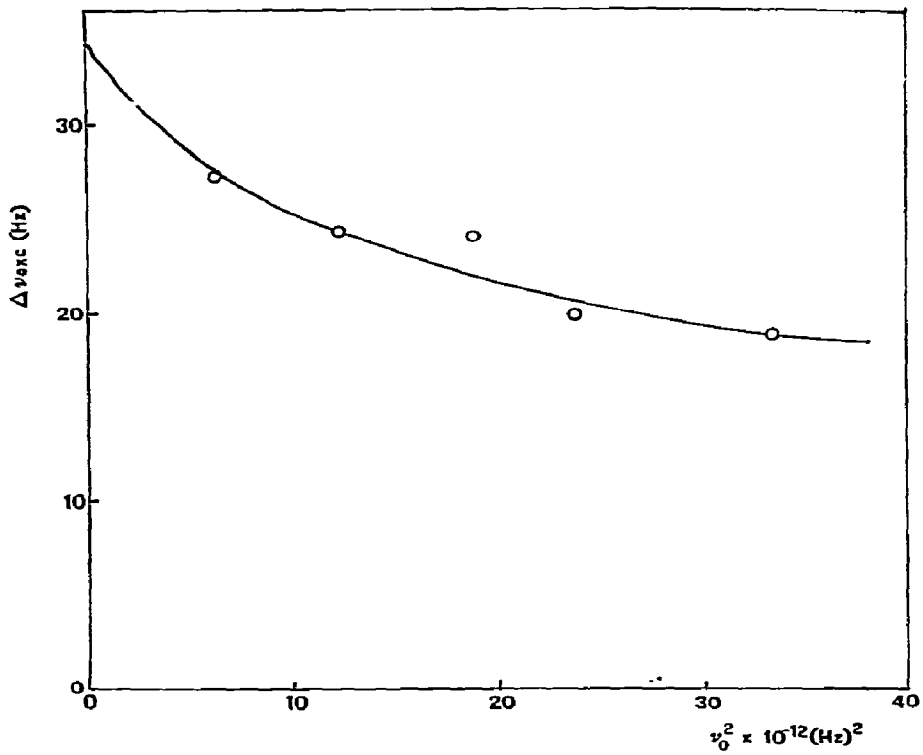

Fig. 4. Frequency dependence of the ^{35}Cl excess linewidth in the presence of Hb Trout IV at ph 6.7. Solvent: 0.5 M NaCl. Hemoglobin derivative: Hb CO at 1.45%.

Eqs. (2), (3) and (4) may be combined to give

$$\Delta\nu_{exc} = \sum_i \frac{n_i [P]_t K_i}{1 + K_i [Cl^-]_t} \times \frac{1}{\pi}\left(\frac{0.6}{T_{2M}^{i'} + \tau_M^i} + \frac{0.4}{T_{2M}^{i''} + \tau_M}\right). \quad (5)$$

Previous work on chloride binding to normal and chemically modified human hemoglobins [4, 5] has shown that the experimental data can be fitted to eq. (5) by assuming the presence of two classes of binding sites, one of high and the other of low affinity sites are oxygen linked. Furthermore the relaxation rates of the bound nuclei are dominated by quadrupole effects [4]. Our present data on the dependence of linewidth on chloride concentration may be fitted satisfactorily assuming only one class of high affinity sites in Hb-Trout I (cf. fig. 1a); on the other hand in Hb-Trout IV an additional term for the presence of a class of low affinity is required (cf. fig. 1b).

Different criteria have been used to decide whether the observed linebroadening is determined by chemical exchange or quadrupole effects: they involved the determination of the temperature and frequency dependence of the excess linewidth and the measurement of the linewidth for the two chloride isotopes, ^{35}Cl and ^{37}Cl.

In the study of the temperature dependence [9] if chemical exchange determines the linewidth ($\tau_M \gg T'_{2M}, T''_{2M}$) the linewidth will increase with increasing temperature since τ_M is bound to decrease; on the other hand if the observed relaxation rate is not limited by the chemical exchange process ($\tau_M \ll T'_{2M}, T''_{2M}$) the linewidth will decrease with increasing temperature since, according to eqs. (4), (4a) and (4b), $1/T_2^i$ is roughly proportional to τ_c. For an approximately spherical molecule τ_c is proportional to η/T, where η is the viscosity of the medium and T the absolute temperature.

Table 2
Excess linewidth for the two chloride isotopes ^{37}Cl and ^{35}Cl in 0.5 M NaCl solutions containing Hb Trout I and Hb Trout IV

Component	Derivative	$\Delta\nu_{exc}(^{35}Cl)$ (Hz)	$\Delta\nu_{exc}(^{37}Cl)$ (Hz)	$\frac{\Delta\nu_{exc}(^{35}Cl)}{\Delta\nu_{exc}(^{37}Cl)}$ [a]
Hb Trout I (0.34%)	deoxy	25.0	22.2	1.1
	CO	13.9	14.2	1.0
Hb Trout IV (1.6%)	deoxy	16.5	12.2	1.4
	CO	22.2	13.6	1.6

a) The estimated error in this ratio is ± 0.1.

The results obtained for Hb-Trout I and Hb-Trout IV (fig. 3) show an oposite temperature dependence of the linewidth: for Hb-Trout IV the observed relaxation appears to be dominated by the chloride relaxation rate at the binding site, as previously observed for human hemoglobin and other chemically modified hemoglobins [4; 5]. However in the case of Hb-Trout I, the linewidth seems to be controlled by the rate of chemical exchange. This result is most unexpected since slow exchange has never before been encountered in proteins which do not contain metals in the Cl^- binding site. In order to ensure that slow exchange was not due to metal contamination the temperature dependence was checked with different preparations of Hb-Trout I and in the presence of complexing agents (EDTA, BAL).

An independent check on the factor controlling the overall relaxation rate is provided by the study of the linewidth for the two chloride isotopes ^{35}Cl and ^{37}Cl. From eq. (5) it follows that when $\tau_M \ll T'_{2M}, T''_{2M}$, i.e., when quadrupole relaxation is dominating, the ratio of the linewidth for the two chloride isotopes should follow the equation*

$$\frac{\Delta\nu_{exc}(^{35}Cl)}{\Delta\nu_{exc}(^{37}Cl)} = \left(\frac{Q(^{35}Cl)}{Q(^{37}Cl)}\right)^2 = 1.61, \qquad (6)$$

where Q is the quadrupole moment of the nucleus [16]. When, however, $\tau_M \ll T'_{2M}, T''_{2M}$, i.e. when chemical exchange is dominating, the ratio of the linewidth for the two chloride isotopes should equal

$$\frac{\Delta\nu_{exc}(^{35}Cl)}{\Delta\nu_{exc}(^{37}Cl)} = \frac{\tau_M(^{35}Cl)}{\tau_M(^{37}Cl)} = 1.0, \qquad (7)$$

disregarding the small possible isotope effects on the exchange rate. In intermediate cases the ratio of the linewidth may take values between these two extremes and thus serve as a measure of the relative contribution of T_{2M} and τ_M to the observed relaxation rate.

The experimental ratios reported in table 2 confirm that chemical exchange limits the observed relaxation rate of the chloride ions in the presence of both the deoxy- and carbonmonoxide-derivatives of Hb-Trout I. In the case of Hb-Trout IV on the other hand, the relaxation at the binding site(s) is dominating.

Additional proof that quadrupole effects prevail in the case of Hb-Trout IV is given by the frequency dependence of the chloride linewidth (fig. 4). The experimental data for ^{35}Cl could be satisfactorily fitted with eq. (5) assuming that the exchange lifetime is much smaller than the quadrupole relaxation time at the binding site(s). The value of $\tau_c = 3.2 \times 10^{-8}$s obtained from a least squares fit of eq. (5) to the experimental data is in fair agreement with the data reported in the paper by Bull et al. [11]. The frequency dependence of the linewidth shown in fig. 4 further indicates that at the normal operating frequency of our NMR instrument, 5.8 MHz, the experiments are made close to extreme narrowing conditions.

The body of experimental data just discussed gives conclusive evidence that in Hb-Trout I the chloride binding site(s) shows slow chemical exchange and hence must be of a rather unusual nature.

In a simple binding process τ_M equals $1/k_{off}$; and for $K[Cl^-] \gg 1$ the excess linewidth should have a $1/[Cl]$ dependence (see eq. (5) and ref. [8]) as appears to be

* Eq. (6) is valid under conditions of non-extreme narrowing only if the linewidth of the two isotopes are determined at the same frequency of the observing r.f. field – as was the case in our experiments.

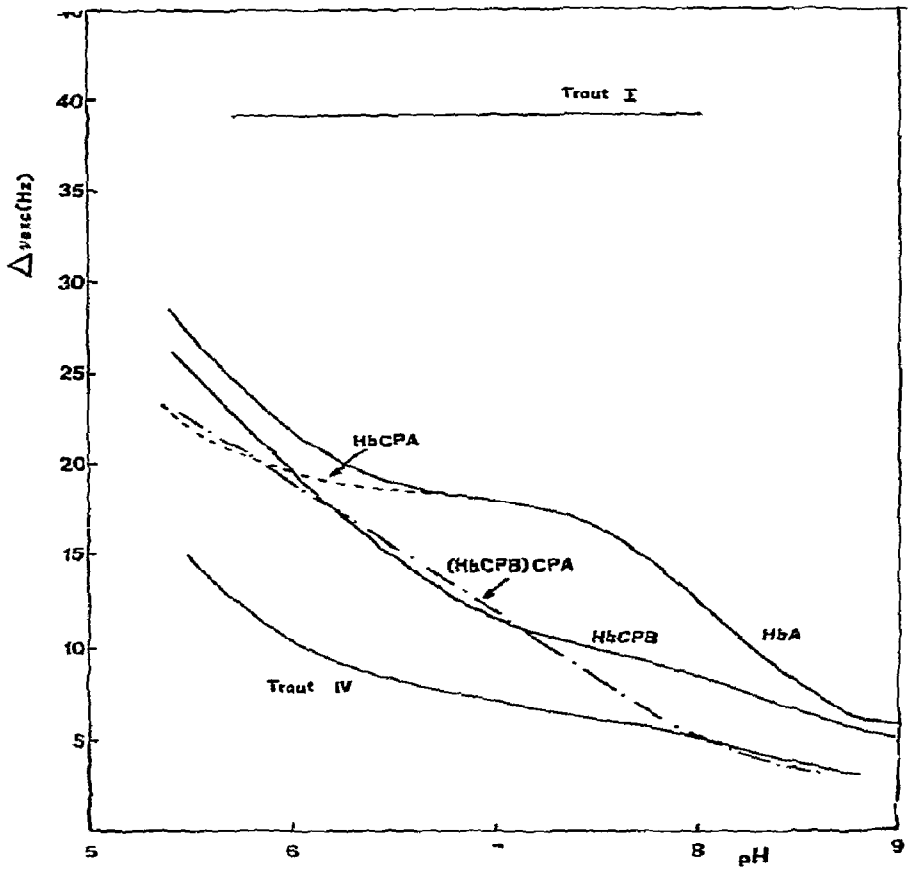

Fig. 5. Excess linewidth as a function of pH in the presence of normal and chemically modified human hemoglobin, Hb Trout I and Hb Trout IV. The lines for human hemoglobin were taken from Chiancone et al. [5] and refer to the carbonmonoxide derivative. The excess linewidth is normalized to a protein concentration of 1% in 0.5 M NaCl. HbA, normal hemoglobin; HbCPA, hemoglobin digested with carboxypeptidase A (des his β-146, tyr β-145); HbCPB, hemoglobin digested with carboxypeptidase B (des arg α-141); (HbCPB)CPA, hemoglobin digested sequentially with carboxypeptidase B and A (des arg α-141, tyr α-140, his β-146, tyr β-145).

the case for Hb-Trout I (fig. 1a). The linewidth data presented in fig. 1a may therefore be used to calculate a value of τ_M, or rather a value of τ_M/n where n is the number of chloride ions bound, through a simple rearrangement of eq. (5) for a single class of sites in the slow exchange case:

$$\frac{1}{\Delta\nu_{exc}} = \left(\frac{1}{K} + [Cl^-]\right)\frac{\pi\tau_M}{n[P]_t}. \qquad (8)$$

From a plot of $1/\Delta\nu_{exc}$ versus $[Cl^-]$ we obtain $\tau_M/n = 2.6\,(\pm 0.6) \times 10^{-6}$s for the carbon monoxide derivative of Hb-Trout I. A similar plot of deoxy Hb-Trout I (data taken from fig. 1a) gives $\tau_M/n = 1.6\,(\pm 0.6) \times 10^{-6}$s.

The interpretation of the slow exchange rate in terms of structural features of the molecule can at this stage be only tentative. One possibility is that we observe chloride binding at the organic phosphate binding site. We may assume that the organic phosphate binding site is in the same region in all tetrameric hemoglobins, i.e., on the dyad axis between the β-chains [12]. The investigation of the oxygen

binding properties in the presence of different anions has shown that chloride and orthophosphate affect the oxygen affinity of Hb-Trout I. On the other hand organic phosphates, such as ATP, are unable to induce any change in the ligand binding properties [13]. Findings of this type suggest that the anion binding area in the hemoglobin molecule is easily accessible to the smaller effectors, and this is supported by the lack of competition between organic phosphates and chloride. On the basis of these considerations one may propose that the strong chloride binding site(s) in Hb-Trout I is located in a crevice not freely interacting with larger affectors both in oxy and deoxy Hb. This may provide a clue to the interpretation of the small chloride dissociation constant k_{off}. Neglecting uncertainties in the number of bound chlorides the value of τ_M is about hundredfold larger than that characteristic of fast exchange proteins (see human hemoglobin). This difference leads to an activation free energy of about 10 kcal/mole as calculated from the theory of absolute reaction rates. The experimental value of the activation enthalpy for the linebroadening is 5.5 kcal/mole. Although the linewidth for the Trout I Hb is dominated by the chemical exchange rate, a small and temperature dependent contribution from the T_{2M} term in eq. (5) may give too low a value for the true activation enthalpy for the temperature variation of τ_M. The entropy of activation $\Delta S^{\ddagger}$ of about -13 e.u. for the exchange process, calculated from the theory of absolute reaction rates, must then be considered as a minimum value.

It should also be pointed out that $\Delta\nu_{exc}$ is pH independent from 5.7 to 8.0 (cf. fig. 2a). This finding is fully consistent with the absence of pH effects on the functional properties of Hb-Trout I [1, 2]. Although it cannot be excluded that the number of strong binding sites changes with pH it seems most likely that both n and τ_M remain constant with pH. Thus the reduction in τ_M/n observed upon deoxygenation may be attributed to a ligand linked structural change which influences the accessibility of the crevice.

In Hb-Trout IV, although the chloride ions are fast exchanging as in human hemoglobin, the detailed characteristics of the binding process are different. The pH profile of the linewidth is reminiscent of that of human hemoglobin, although the absolute values and the pH dependence above pH 7 are much less marked. The observed pH profile, when compared with other available data (fig. 5) allows some tentative structural considerations to be made. Previous work suggested that the C-terminal region of both the α- and β-chains and the N-terminal Val of the α-chains are involved in chloride binding. On the other hand from the primary structure of Hb-Trout IV it is known that: (i) the C-terminus of the β-chains is His, but that His-143 is substituted by arginine; (ii) the N-terminus of the α-chains is a blocked serine (probably Acetyl-Ser) [14]. Both these modifications should introduce structural perturbations in the cledged chloride binding regions and may explain the observed differences among Trout IV and human hemoglobin. Since oxygen binding as well as pH changes are associated with tertiary and quaternary structural changes (see [1] for review), the findings reported above imply that chloride is probing localized region(s) of the Hb-Trout IV molecule not affected by these changes. In particular the role of the terminal amino group of the α-chains in chloride binding is confirmed by the present data. Thus the lack of a marked inflection in the pH dependence above 7, and independence of linewidth on O_2 binding may both depend on the unavailability of the α-NH_2 group of the α-chains which in Hb-Trout IV is blocked.

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