^{35}Cl NMR STUDY OF THE RELEASE OF CHLORIDE ON OXYGEN BINDING TO HUMAN HEMOGLOBIN

Jan-Erik NORNE, Emilia CHIANCONE, Sture FORSÉN, Eraldo ANTONINI and Jeffries WYMAN

Consiglio Nazionale delle Ricerche Center of Molecular Biology, Institutes of Chemistry and Biochemistry, Faculty of Medicine, University of Rome, 00185 Rome, Italy and *Department of Physical Chemistry, Chemical Center, POB 740, Lund Institute of Technology, S-22007 Lund, Sweden*

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1. Introduction

NMR quadrupole relaxation studies involving ^{35}Cl have proved to be a sensitive tool for the characterization of the interaction of Cl^- with human hemoglobin. Thus, direct evidence has been obtained for the differential binding of chloride to oxy- and deoxyhemoglobin, which accounts for the decrease in oxygen affinity produced by NaCl [1,2]. Specific chloride binding sites on the protein have been identified with the aid of mutant and/or chemically modified hemoglobins. It appears that chloride is bound with high affinity not only at the organic phosphate binding site, situated in the cleft between the β chains, but also at (or near) the C-terminal regions of both types of chain (His β-146–Asp β-94 and Val α-1–Arg α-141) [3,4].

The present work was undertaken to determine the relationship between the fractional saturation with oxygen and that with chloride in concurrent experiments. The measurements were carried out in the presence or absence of organic phosphates.

The results show that the fractional change in ^{35}Cl linewidth is not linearly related to the fractional saturation with oxygen in particular in the absence of organic phosphates. The general shape of the curve agrees well with the dependence of the fraction of molecules in the R state on the fractional oxygen saturation, as calculated according to the Monod-Wyman-Changeux model [5].

2. Materials and methods

Human hemoglobin was prepared from freshly drawn blood by the ammonium sulfate procedure. The protein was freed from inorganic and organic ions by passage through a mixed-bed ion-exchange resin. 2,3-Diphosphoglycerate (DPG) was obtained from Sigma as the pentacyclohexylammonium salt and converted to the acid with Dowex 50-X8; the solution was then neutralized with concentrated NaOH.

Visible and NMR spectra were observed concurrently at various stages of oxygenation using an air-tight NMR tube with a 2 mm cuvet fused to the top. A small side arm, sealed by a rubber septum, allowed injection of air into the tube by means of a hypodermic syringe during the course of the experiments. Optical spectra were recorded with a Hitachi-Perkin-Elmer Model 124 double-beam recording spectrophotometer. ^{35}Cl spectra were measured on a modified Varian XL-100 spectrometer operating in the Fourier transform mode. The linewidths were measured at half-heights of the absorption signals. The temperature of the probe was maintained at 27.4 ± 0.1°C; the hemoglobin solutions were equilibrated at the same temperature after each addition of air.

The pH was measured on a Radiometer 63 pH meter.

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3. Results and discussion

The binding of Cl^- as a function of oxygenation was followed by measuring the ^{35}Cl linewidth for 0.2 M or 0.5 M NaCl solutions in the presence of 1% human hemoglobin at pH 6.4 and 7.0. Under these experimental conditions the broadening of the linewidth of the free ion due to interaction with the protein, $\Delta\nu_{ex}$, is due primarily to high affinity sites and differs significantly in the case of oxy- and deoxyhemoglobin [1]. The fractional change in ^{35}Cl linewidth upon oxygenation, η, can be defined as follows:

$$\eta = \frac{\Delta\nu_{ex}(\bar{Y}) - \Delta\nu_{ex}(O)}{\Delta\nu_{ex}(I) - \Delta\nu_{ex}(O)}$$

where $\Delta\nu_{ex}$ is the observed ^{35}Cl linewidth after subtraction of the linewidth of free Cl^-. Figure 1 shows the fractional change in ^{35}Cl linewidth, η, as a function of the fractional saturation with oxygen, $\bar{Y}$, measured at pH 6.4. At pH 7 the dependence is similar, although the deviation from linearity is less pronounced. The change in ^{35}Cl linewidth is non linear with oxygen saturation and is greatest in the later stages of oxygenation. The lag in chloride release with respect to oxygen binding is thus similar to that of DPG as observed in concurrent visible and ^{31}P NMR spectroscopy experiments [6].

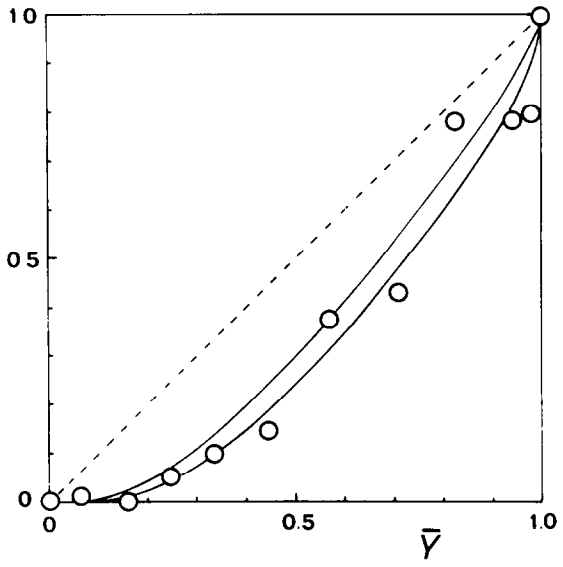

Fig.1. Fractional change in the ^{35}Cl excess linewidth, η, as a function of the fractional oxygen saturation, $\bar{Y}$, in human hemoglobin solutions. Protein concentration: 1% in 0.5 M NaCl, at pH 6.4. The solid lines give the fraction of molecules in the R state calculated with the MWC parameters $L = 2$ and 5×10^6, $c = 10^{-2}$.

Many features of the cooperative behaviour of the hemoglobin molecule can be accomodated in the framework of the two-state allosteric model of Monod-Wyman-Changeux [5]. Along this line, the simplest interpretation of the ^{35}Cl NMR data of fig.1 is that the change in excess linewidth, as we go from the deoxy (T) to the oxy (R) form of hemoglobin, is directly proportional to the fraction of molecules in the R state. In other words the Cl^- is probing the overall conformation of the hemoglobin molecule. The midpoint of the T⟶R transition should then according to fig.1 take place when $\bar{Y} = 0.7$. This value agrees fairly well with that calculated from the allosteric two-state model. Normal conditions for ligand binding to hemoglobin correspond to $L = 10^5 - 10^6$ and $c = 10^{-2}$. With $L = 2-5 \times 10^6$ and $c = 10^{-2}$ we obtain the transition at $\bar{Y} = 0.65-0.70$. The general shape of the η versus $\bar{Y}$ curve agrees well with the dependence of R on $\bar{Y}$ according to the Monod-Wyman-Changeux model which is illustrated by the solid lines in fig.1. The ^{35}Cl NMR data as a function of oxygenation appear to be consistent with conclusions drawn from detailed oxygen equilibrium experiments which indicate that in 0.2–0.5 M NaCl solutions at pH 7.4 and 25°C most of the chloride is released at the third oxygenation step (Imaizumi, Imai and Tyuma, in preparation).

Similar studies of the fractional change in ^{35}Cl excess linewidth as a function of fractional oxygen saturation were also carried out in the presence of DPG in high excess (25–50 mM). Under these conditions, when the Cl^- has been expelled from the DPG binding site, there is still oxygen-linked chloride release as indicated by the small, but significant decrease in oxygen affinity as a function of chloride concentration (table 1). The fractional change in ^{35}Cl linewidth, η, obtained in the presence of 25 mM DPG at pH 6.4 is shown in fig.2. The observed dependence of the linewidth parameter, η, on $\bar{Y}$ does not seem to fit with the predictions from the two-state model. The effect of the addition of DPG is to

Table 1
Oxygen equilibrium of human hemoglobin as a function of NaCl concentration in the presence of 25 mM DPG at pH 7.2 and pH 6.5

pH	NaCl (M)	log $p_{1/2}$
7.2	0	0.87
7.2	0.05	0.90
7.2	0.5	0.98
6.5	0	1.10
6.5	0.5	1.20

Hemoglobin 10 mg/ml; temp. 20°C

stabilize the T-form raising the value of the parameter L. We thus expect the mid-point of the T⟶R transition in the presence of DPG to shift towards somewhat larger values of $\bar{Y}$ than in its absence. In the curve of fig.2, however, the value of $\eta = 0.5$ is obtained at somewhat lower $\bar{Y}$ values than in fig.1. We must remember that in the presence of DPG in excess CL^- is probing different anion binding sites (most likely the region around Arg α-141 and Val α-1) and that these sites might reflect more local rather than overall conformational changes of the hemoglobin molecule. This interpretation is also consistent with the results of Cl^- binding experiments on carboxypeptidase-digested hemoglobins [3].

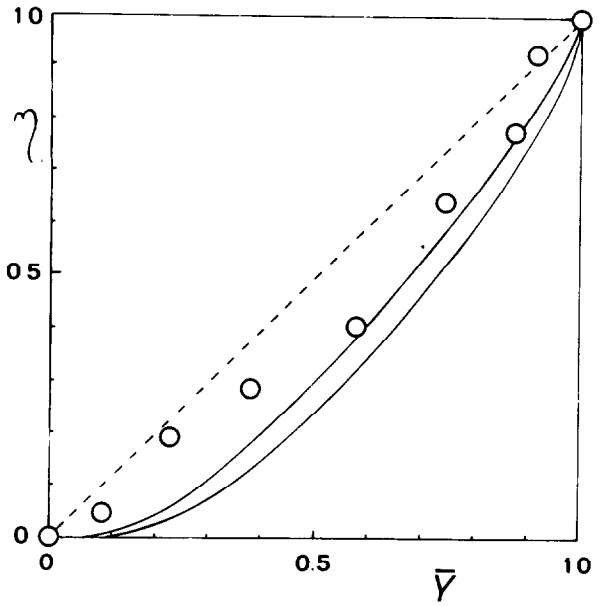

Fig.2. Fractional change in the ^{35}Cl excess linewidth, η, as a function of the fractional oxygen saturation, $\bar{Y}$, in human hemoglobin solutions containing 2,3-DPG in excess. Protein concentration: 1% in 0.5 M NaCl and 25 mM DPG, at pH 6.4. The solid lines give the fraction of molecules in the R state calculated with the MWC parameters $L = 2$ and 5×10^6, $c = 10^{-2}$.

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