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^{19}F NMR STUDY OF THE INTERACTIONS OF FLUORIDE WITH SUPEROXIDE DISMUTASE AND HEMOGLOBIN IN ERYTHROCYTES

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F^- added to an erythrocyte suspension shows two separated resonances arisen from intra and extracellular compartments. Cu,Zn superoxide dismutase dominates the longitudinal relaxation rate of the intracellular F^- resonance, while diamagnetic interactions with hemoglobin contribute mainly to the transversal relaxation rate.

NMR measurements in whole cells or in living organisms provide specific information about molecular structure, equilibria and dynamics of biological systems. However NMR observation of nuclei such as ^1H could be difficult because of the overlapping of the signals. To this regard, fluorinated molecules appear useful NMR probes for the absence of endogenous resonances, together with the ^{19}F high sensitivity and large chemical shift range. In this paper we report the results of a preliminary study utilizing $^{19}F^-$ as a probe for the interaction with macromolecules in erythrocytes. This anion appears suitable because it easy permeates the cell membranes, probably through the Band 3 transporter (1) and it was shown to be an interesting probe of paramagnetic binding sites (2). Aim of this study is to elucidate the physical interaction controlling the chemical shift and the relaxation rate of F^- in erythrocytes.

MATERIALS AND METHOD

The chemicals (Aldrich) were of the best available purity, and were used without further purification. The solutions were prepared in twice distilled water. The gases were from SIAD (Padova). Venous blood was obtained from adult healthy volunteers, according to the Helsinki declaration, using EDTA as anticoaugulant.

Abbreviations
PBS, potassium buffered saline; BSA, bovine serum albumin; DDC, diethyldithiocarbamate; Ht, hematocrit.

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The erythrocytes were washed three times with PBS containing 1 g/l of glucose and stored at 4°C for less than 10 hours. Before each experiment the cells were incubated for 15 min in a solution containing 10% PBS, 50% D_2O, 5 x 10^{-2} - 10^{-1} M F^-. NaCl was added to get isotonicity. The pH of the solutions was adjusted to 7.4 before the addition of D_2O. After incubation, the pH-pD of the supernatant was controlled and the shift from pH 7.3 was usually less than 0.1 unit. The cells were centrifuged at 12,000 x g for 15 min at 4°C and after the discharge of the supernatant the hematocrit was about 98%. The lysis was usually negligible. The experiments at different hematocrit were performed diluting the packed cells in varying proportion with the supernatant solution. The hemoglobin (Hb) concentration, calculated from the absorbance at 577 nm (ε = 14.6 x 10^3 M^{-1} cm^{-1}, being 16,000 the equivalent Hb molecular weight) (3), was taken as the measurement of the cell dilution. The absorbance measurements were performed by a Perkin-Elmer Lambda 17 UV-VIS spectrophotometer.

The experiments at different pO_2 and pCO were carried out by gently bubbling the erythrocyte suspensions with humidified N_2, air, O_2, CO just before sealing the NMR tube. The hemolysates were prepared by freezing and thawing cycles or by addition of Triton X-100 (0.5% w/w).

When necessary the paramagnetic contribution of Cu,Zn superoxide dismutase to the ^{19}F relaxation rate was made negligible by incubation with 10^{-2} M diethyldithiocarbamate (DDC) at 37°C for 1h (4), or by equilibration of the erythrocytes with a large volume of $5x10^{-2}$ M KCN in PBS, buffered at pH 7.4.

The NMR spectra were recorded on a Bruker MSL 300 operating at 282 MHz for the ^{19}F. The experiments were usually performed in 5 mm tubes with a co-axial capillary containing pentafluoropyridine (2% in $CHCl_3$) as chemical shift reference. The central peak of the quintet at higher field of the pentafluoropyridine spectrum was taken as reference. The spinning rate was 10 Hz. The relaxation rates measurements were performed in 10 mm tubes, without spinning and reference, by automation microprograms, using the inversion recovery sequence for the T_1 and CPGM sequence for the T_2 respectively (5). In some experiments the T_2 was measured by the half-height linewidth.

RESULTS

The addition of increasing amount of an erythrocyte lysate equilibrated with air to a solution containing $5x10^{-2}$ M F^-, buffered at pH 7.3, affects both the chemical shift and the half-height linewidth of the F^- resonance. Fig. 1 shows the chemical shift of the F^- as a function of the Hb concentration, being Hb mainly in the oxy form (98%). It appears that the F^- resonance shifts linearly toward downfield increasing the lysate concentration. The addition of bovine serum albumin (BSA) to an F^- solution produces a similar downfield shift (see Fig. 1). Under the same experimental conditions the $^{19}F^-$ half-height linewidth increases linearly with the lysate concentration in the range [Hb] = 0-230 gL^{-1}. The data are fitted by the equation: $\Delta\nu_{1/2}$ = 1.432 + 0.330 [Hb], correlation coefficient 0.998, where $\Delta\nu_{1/2}$ (Hz) is the half-height linewidth. A similar broadening of the F^- resonance was also observed in the presence of BSA. The $^{19}F^-$-NMR spectrum of erythrocytes

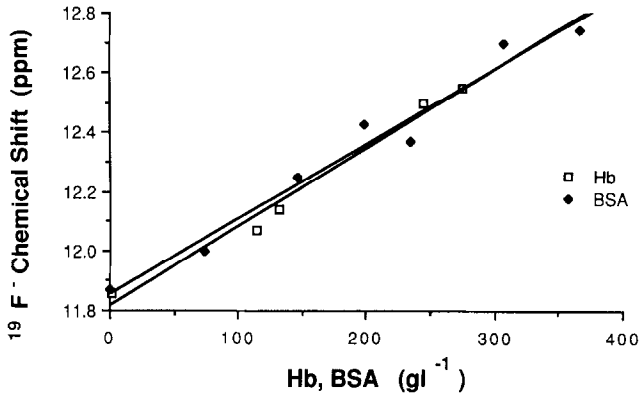

Fig. 1. $^{19}F^-$ chemical shift as a function of the concentration of hemoglobin and of bovine serum albumin. The solution contained 5 x 10^{-2} M F^-, 30% D_2O, 10% PBS and NaCl to obtain physiological ionic strength. (□) Hb (erythrocyte lysate); (♦) BSA.

(Fig. 2) suspended in an isotonic medium, containing 5x10^{-2} M F^-, shows two separated resonances characterized by different half-height linewidth. Taking into account that the fluoride ion enters the erythrocyte membrane (6), the two signals should arise from the intra and extracellular compartments. In fact, the sharper upfield peak at about 11.3 ppm disappears after addition of 10^{-4} M Mn^{2+}-aquo, which is a strong extracellular relaxing agent (7). The shift between the two peaks is almost independent of the hematocrit (Ht) in the Ht range 19%-98%.

The effect of the Hb iron spin state on the spectral characteristics of the F^- ion was assayed equilibrating the erythrocytes suspension with N_2, CO, air and O_2. No

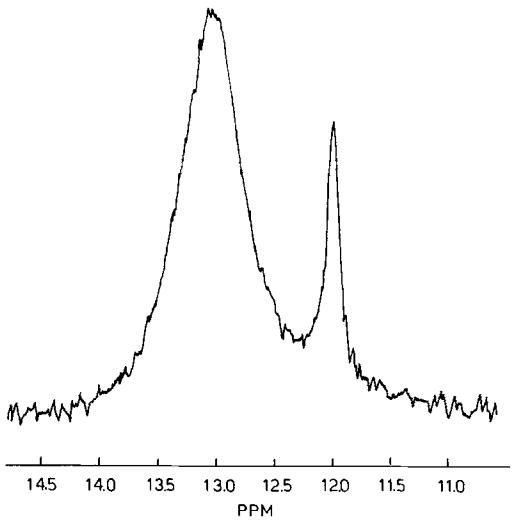

Fig. 2. $^{19}F^-$ spectrum in a suspension of whole erythrocytes.
The erythrocytes were incubated in an F^- solution (see Methods). The final F^- concentration inside the cells was about 5 x 10^{-2} M. Ht 95%.

significative change of chemical shifts and the half-height linewidth were observed for the intra and extracellular $^{19}F^-$ resonances.

The intracellular longitudinal, $(T_1)^{-1}$, and transversal, $(T_2)^{-1}$, relaxation rates of F^- in packed erythrocytes from three different donors resulted in the range 15-25 s^{-1} and 150-300 s^{-1}, respectively and were independent of Ht. On the other hand, $(T_1)^{-1}$ and $(T_2)^{-1}$ of the extracellular F^- were found to be dependent on hematocrit only for high Ht values (Ht >70%).

We assayed the effect of Cu,Zn superoxide dismutase on the F^- intracellular resonance. In fact, this enzyme, present inside the erythrocytes in the concentration range 1-3 μM and containing Cu^{2+} into the active site, strongly affects the relaxation rates of the F^- (8). To evaluate the contribution of this enzyme to F^- relaxation rates in erythrocytes, we measured $(T_1)^{-1}$ and $(T_2)^{-1}$ in the presence of DDC or of cyanide. Both these copper ligands, when present at millimolar concentration (8,9), fully inhibit (≥98%) the activity and the paramagnetic contribution to the F^- relaxation rate of the Cu,Zn superoxide dismutase. The results obtained, showed in Table 1, indicate that the contribution of Cu,Zn superoxide dismutase to $(T_1)^{-1}$ and $(T_2)^{-1}$ of F^- are about 98% and 40%, respectively.

The temperature dependence of the transversal relaxation rate of F^- was measured from the half height linewidth in erythrocyte lysates in the presence and in the

TABLE 1
Relaxation rates of $^{19}F^-$ within whole erythrocytes: Effect of Cu,Zn superoxide dismutase inhibitors

Addition	$(T_1)^{-1}/\ [(T_1)^{-1}]_0$ %	$(T_2)^{-1}/[(T_2)^{-1}]_0$ %
none	100	100
DDC 5×10^{-2} M	2.7	60.5
CN^- 5×10^{-2} M	0.13	62.9

The relaxation rates were measured for the intracellular resonance and were corrected for the relaxation rate of F^- in the buffer solution.
$[(T_1)^{-1}]_0$ and $[(T_2)^{-1}]_0$ indicate the relaxation rates before the addition of superoxide dismutase inhibitors.
Experimental conditions: F^- 5×10^{-2} M, Ht 98%. For the equilibration of the cells with F^- and CN^-, see Methods.

absence of DDC. In both cases it was found that the $(T_2)^{-1}$ increases with the temperature (T). In particular, the plots of ln $[(T_2)^{-1}]$ against $(T)^{-1}$ are linear, suggesting that the F^- transversal relaxation rate is controlled by a slow exchange process. From these plots an activation enthalpy of -6.8 Kcal $mole^{-1}$ was calculated, both in the presence and in the absence of DDC.

DISCUSSION

The increase of the chemical shift and the high relaxation rates of F^- we have observed in whole erythrocytes or in their lysates clearly indicate a variety of physical interactions of the F^- in the intracellular environment. In particular, the enhancement of $(T_1)^{-1}$ can be ascribed to the interaction between F^- and the superoxide dismutase, as shown by the experiments with DDC and CN^-. In fact this enzyme was demostrated to be a strong F^- relaxing agent (8). On the contrary, the Cu,Zn superoxide dismutase is responsible for only the 40% of $(T_2)^{-1}$. The large chemical shift and the high residual transversal relaxation rate can be ascribed to the exchange of F^- between binding sites on proteins, namely Hb, and the bulk. The lack of effects of hemoglobin iron spin-state on F^- resonance suggests that, at difference from phosphate anions (10,11), the heme group does not control the spectral characteristics of F^-. Therefore, the chemical shift and the $(T_2)^{-1}$ of F^- in erythrocytes are controlled by the diamagnetic interactions between F^- and the slow tumbling Hb mojety. This is in agreement with the findings of Hills et al. who observed an increase of transversal proton relaxation rate of solutions of native BSA (12). Moreover, the F^- chemical shift increase we have found in the presence of BSA supports this behaviour. In conclusion, it is reasonable to suppose that F^- interacts with positively charged groups on the Hb surface. To this regard, early studies on Hb-anion interactions suggested Hisβ146-Aspβ94 and Valα1-Argα14 as the groups involved in Cl^- binding (13).

The increase of the transversal relaxation rate with temperature, when the Cu,Zn superoxide dismutase paramagnetic contribution is vanished by DDC, suggests that $(T_2)^{-1}$ is governed by the exchange rate between the bulk and bound sites (14). This result and the ratio $(T_2)^{-1}/(T_1)^{-1} \gg 1$, on the basis of Swift and Connick equations (15), indicate that the interaction between F^- and Hb is characterized by $T_{1M} > \tau_M > T_{2M}$, being τ_M, T_{1M} and T_{2M} the half-life time and the longitudinal and transversal relaxation rates of F^- in the bound site. Since the hemoglobin correlation time (τ_c) is $\tau_c \geq 5 \times 10^{-8}$ s (16), the term $(\omega\tau_c)^2$ is ≥ 100, where ω is the Larmor frequency. On

the basis of the Swift and Connick equation (15), $(\omega\tau_c)^2 \geq 100$ accounts for the high $(T_2)^{-1}/(T_1)^{-1}$ ratio.

The dynamic binding of F^- to diamagnetic sites explains also the linear increase of F^- chemical shift with increasing lysate concentration. Finally, the difference of the protein content between intra and extracellular compartments determines the transmembrane chemical shift we observe when F^- is present in an intact erythrocyte suspension in saline buffer. In fact the chemical shift of the intracellular peak is comparable to that measured in the lysate of packed erythrocytes.

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