Nuclear Magnetic Relaxation Spectroscopy in Solutions of Bovine Hemoglobin

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The longitudinal proton relaxation time $T_1$ of aqueous solutions of bovine ferrihemoglobin and carboxilated hemoglobin was measured in the Larmor frequency range $3 \times 10^5$ Hz to $1,2 \times 10^6$ Hz. The dispersion curves are explained by diamagnetic and electron paramagnetic relaxation mechanisms. An efficient direct dipolar coupling between the paramagnetic heme-groups and the water protons in ferrihemoglobin solutions can be excluded. The flat dispersion behaviour of the diamagnetic contribution suggests a broad distribution of molecular motions i.e. a certain flexibility of the hemoglobin molecule.

Highly concentrated solutions of proteins show a characteristic dispersion behaviour of the longitudinal proton relaxation time $T_1$, as discussed in several papers. In these papers a low-frequency dispersion of $T_1$ at about $10^5 - 10^6$ Hz could be explained by rotational diffusion of the protein molecule. The high-frequency dispersion at $10^6 - 10^7$ Hz may be caused by the motion of movable protein parts as side groups or chain segments.

At even higher frequencies relaxation processes were detected by dielectric relaxation: one process at $10^7 - 10^9$ Hz, which was attributed to hindered motion of bound water, and the well known relaxation process of the unbound water ($10^{10}$ Hz). In the case of hemoglobin solutions dielectric measurements deliver also a clear low-frequency process, which is explained as mentioned above, while ultrasonic absorption measurements show a non-resolved broad distribution of relaxation processes over almost the whole in l.c. investigated frequency range (0,5 to 1000 MHz). At this situation it seemed to be valuable to introduce nuclear magnetic relaxation dispersion measurements into hemoglobin research as a new method.

Experimental procedure

The measurements of the proton longitudinal relaxation time $T_1$ were performed in a range of Larmor frequency $\nu_L = 3 \times 10^5$ Hz to $1,2 \times 10^6$ Hz. Above $4,5 \times 10^5$ Hz a conventional NMR-pulse-spectrometer was used, while the low-frequency range down to 3 kHz was reached by the field-modulation technique as described in l.c. The mean square measuring error was about 5 percent.

The samples were prepared using bovine hemoglobin (Fluka, 2x cryst., 100%), and borate buffer (Merck, pH 9,22). The concentration of the solutions was 0,25 g vacuum dried hemoglobin per cm² buffer solution.

In order to reveal the relaxation effect of the paramagnetic heme-groups in ferrihemoglobin (Hb(H₂O), S = 5/2), a series of measurements with diamagnetic carboxilated hemoglobin (HbCO, S = 0) was performed. In this case a high percentage of carboxihemoglobin in the solutions was ensured by putting the ferrihemoglobin samples under 80 atm carbonmonoxide for about 70 h. After saturation of the HbCO-contents, as indicated by constant T₁-values, the samples were cautiously frozen in the CO-atmosphere and sealed in sample tubes. This preparation procedure delivered reproducible samples within the measuring error. Thus two series of experiments with the same samples but different hemoglobin modifications could be performed. The ratio between the relaxation times in ferrihemoglobin and carboxilated hemoglobin solu-

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Results and Discussion

In Fig. 1 the $T_1$-dispersion curves of the hemoglobin solutions are shown. A significant feature of the ferrihemoglobin solutions is their flat dispersion compared to diamagnetic protein solutions $^{1-6}$. One could assume that this different behaviour is due to the electron paramagnetic heme-groups. But the comparison between the low-frequency relaxation times of ferrihemoglobin and serum-albumin $^3$ solutions respectively — serumalbumin has approximately the same molecular weight as hemoglobin — shows that the paramagnetic effect is only a partial relaxation mechanism: The low-frequency relaxation times are related as the inverse protein concentrations in good approximation.

The paramagnetic relaxation effect can be regarded separately by using the results obtained in the electron paramagnetic heme-groups. But as shown in l.c. $^4$ there are spin diffusion processes active within protein molecules leading to a similar behaviour of the protein protons. So eq. (1) describes the measured effect in good approximation.

$$\frac{1}{T_1^{Hb(H_2O)}} = \frac{1}{T_1^{Hb(CO)}} + \frac{1}{T_1^{HbCO}} \quad (1)$$

where $T_1^{Hb}$ is the relaxation rate due to the diamagnetic relaxation processes, and $T_1^{HbCO}$ is the rate caused by the four paramagnetic heme-groups per ferrihemoglobin molecule.

Eq. (1) holds not for the protein protons. But as stated for $T_1$. The relaxation time of the carboxilated hemoglobin solution, one obtains for the paramagnetic contribution

$$\frac{1}{T_1^{Hb(CO)}} = \frac{1}{T_1^{Hb(H_2O)}} - \frac{1}{T_1^{HbCO}} \quad (2)$$

The relaxation time $T_1^{CO}$ versus the proton Larmor frequency $\nu_p$ is plotted in Fig. 2. (It should be recognized that the error of $T_1^{CO}$ lies between 20% at the highest frequencies and 100% at the lowest frequencies as a consequence of the measuring error of 5% for both $T_1^{Hb}$ and $T_1^{HbCO}$). Obviously no dispersion exceeding the measuring error can be stated for $T_1^{CO}$.

For the correlation time $\tau_{dip}$ of the dipolar coupling between a proton and an electron paramagnetic heme-group holds $^{14}$

$$\frac{1}{\tau_{dip}} = \frac{1}{\tau_r} + \frac{1}{\tau_s} + \frac{1}{\tau_e} \quad (3)$$

($\tau_r$, rotational jump time of the electron — proton distance-vector, $\tau_s$ longitudinal electron relaxation time, $\tau_e$ exchange time of the proton spin orientation). The shortest of the three times $\tau_r$, $\tau_s$, and $\tau_e$...
determines the correlation time $\tau_{\text{dip}}$. Assuming that $\tau_r$ is equal to the rotational jump time of the whole hemoglobin molecule, $\tau_r$ should be of the order $10^{-7}$ sec as measured by dielectric relaxation. $\tau_8$ is delivered by ESR-measurements in an order of $10^{-10}$ sec, while $\tau_e$ is expected to be very much greater than this time regarding the possible proton spin transport mechanisms: spin diffusion and water or proton exchange in the solvent. It follows $\tau_{\text{dip}} \sim \tau_8$.

The comparison of the $T_1^p$-curves at 0 °C and 30 °C (Fig. 2) shows that $\tau_s(0 \, ^\circ C) < \tau_s(30 \, ^\circ C)$ because of $T_1^p(0 \, ^\circ C) > T_1^p(30 \, ^\circ C)$ according to the theory of SOLOMON. With other words, the $\tau_s$-values are situated on the frequency-independent “high-temperature branch” of the $\tau_s$ versus temperature curve.

A further dispersion effect could arise at the frequencies given by $2 \pi v_p \tau_s \sim 1$ and $2 \pi v_e \tau_s \sim 1$ ($v_p$ proton Larmor frequency, $v_e \sim 660 v_p$ electron Larmor frequency). The first dispersion frequency ($v_p \sim 10^9$ Hz) falls outside of the frequency range, while around the second frequency ($v_p \sim 10^8$ Hz) a dispersion step with a theoretical height factor $10/3$ should be localized, provided rapid proton or spin exchange occurs. Despite the rather big error of the $T_1^p$-values (Fig. 2) such a step should be observable. As no dispersion could be identified, the conclusion is that diffusion limited exchange processes reduce the dispersion step to an unobservable height. Therefore no efficient direct dipolar coupling can exist between the electronic paramagnetic heme-group and the rapidly exchanging water molecules of the solvent.

As to the diamagnetic relaxation mechanisms, of broad distribution of molecular motions (from $10^5$ Hz to $10^8$ Hz or more) has to be assumed, in order to explain the flat dispersions of $T_1^\text{HDCO}$. Such a distribution agrees well with ultrasonic absorption measurements.

The slowest diamagnetic relaxation process should have a correlation time between $10^{-7} - 10^{-6}$ sec, caused by rotational diffusion of the whole hemoglobin molecule. On the other hand the diffusion of unbound water molecules is certainly the most rapid process, whose dispersion is expected beyond the upper frequency limit. Between these two limiting processes a variety of further relaxation mechanisms can be imagined (provided that proton or spin exchange occurs). As earlier suggested, the rotation of side groups and kink motions of the peptide chain fall into this range. Furthermore a hindered diffusion of water molecules within the hydration sphere is discussed in l. c. The conclusion for the presented frequency range is that hemoglobin molecules are not completely rigid. Possibly they suffer a certain structural softening during solvation compared to the crystalline state. NMR line spectroscopy seems to indicate similar results.

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