OBSERVATION OF DEUTERIUM-LABELED DIACETYLDEUTEROPORPHYRIN INCORPORATED IN CYANOFERRIMYOGLOBIN BY DEUTERIUM NUCLEAR MAGNETIC RESONANCE

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Summary

Sperm whale apomyoglobin was reconstituted with selectively deuterated D_6-2,4-diacetyldeuterohemin in which the ^2H label was confined to the methyl groups of the acetyl moieties. A single resonance was observed in ^2H NMR of the cyanoferrimyoglobin derivative, with a chemical shift 0.80 ppm downfield of external D_{12}-TMS at pH 6.7. The corresponding chemical shift of D_6-2,4-diacetyldeuterohemin-OMe as the cyanide complex in pyridine-water was 0.96 ppm downfield of external D_{12}-TMS. The prominent HOD peak was well separated at 4.4 ppm downfield. The line width of the porphyrin ^2H resonances in both the protein and free solvent environments yields evidence of considerable rotational freedom of the $-CD_3$ groups about their axes.

It has been found possible to observe by ^2H NMR the signal from 2,4-diacetyldeuterohemin selectively deuterated in the methyl groups of the acetyl moieties (Fig. 1) and incorporated in sperm whale apomyoglobin. Myoglobin may be reconstituted with certain artificial Fe (III)-porphyrins with functional properties similar to those of the normal protein (1). For the present purpose the selective deuteration of the heme was achieved in CH_3OD and D_2SO_4 over a period of 24 hours at room temperature in the dark (2). Spectra are presented showing the ^2H NMR behavior of the cyanoferrimyoglobin form and of the free cyanoferriporphyrin form in pyridine-water solution.

Material and Methods

D_6-2,4-diacetyldeuterohemin-OMe (2) was hydrolyzed to remove the ester function in deuterated solvents according to Fischer et al. (3). Sperm whale

apomyoglobin (4) was treated at 4° with a 1.2 molar proportion of the D_6-2,4-diacetyldeuterohemin taken up in 1 ml of 0.1 N NaOH and immediately diluted tenfold with water. After the pH of the solution had been adjusted to 10.5 it was dialyzed first against cold water and then against phosphate buffer at pH 6.2. The artificial myoglobin was chromatographed on CM50 Sephadex with pH 6.2 phosphate buffer, ionic strength 0.1 M. The purity of the product was checked by cellulose acetate electrophoresis. It was lyophilized after deionization on a column of Rexyn I-300 (Fisher). The protein sample was prepared for the NMR experiments by dissolving an appropriate amount in 3 ml of phosphate buffer ionic strength 0.1 M, at pH 6.7 and then concentrating it by Amicon filtration. A tenfold molar proportion of KCN was added. The concentration of the protein sample was 4.5 to 5 mM. The ^2H NMR experiment was done at 14-16° with a recycle time of 0.211 seconds with 32,768 accumulations. The sample of D_6-2,4-diacetyldeuterohemin-OMe (4 mg) was dissolved in a mixture of pyridine and water (0.75 ml/0.25 ml), containing 4 mg KCN. The recycle time was 0.211 seconds, with 4096 accumulations.

The ^2H NMR measurements were performed at 33.77 MHz with a Varian HR 220 MHz spectrometer equipped for pulsed Fourier transform operation. External D_{12}-TMS was used as reference.

Results and Discussion

Fig. 2A shows the ^2H NMR spectrum of the D_6-diacetyldeuterohemin incorporated in the cyanoferrimyoglobin derivative. The major resonance peak at 0.80 ppm downfield from external D_{12}-TMS represents the ^2H nuclei of the two unresolved $-CD_3$ groups (Fig. 1). The D_{12}-TMS resonance is overlaid in the spectrum for the zero reference. The prominent HOD peak is well separated at 4.4 ppm downfield. The pH of the solution was 6.7.

Fig. 2B shows the corresponding spectrum of the small molecule D_6-2,4-diacetyldeuterohemin-OMe as the cyano derivative in pyridine-water, in which the prominent ^2H resonance occurs at 0.96 ppm downfield of external D_{12}-TMS.

The chemical shifts in both cases for these low-spin Fe (III) heme com-

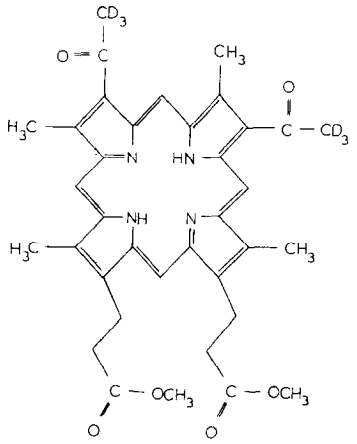

Figure 1. Formula of D_6-2,4-diacetyldeuteroporphyrin-OMe showing the location of the methyl groups substituted with deuterium in the acetyl moieties. The Fe (III) derivative is used for the present study (2).

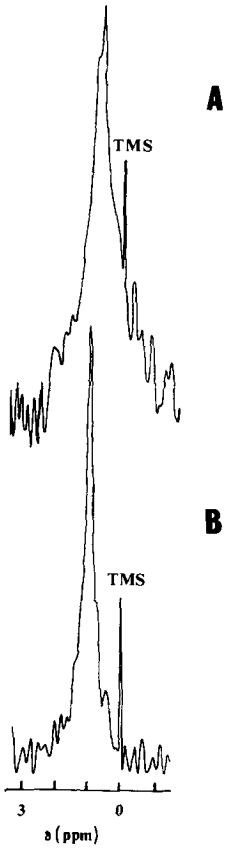

Figure 2. Spectra obtained by pulsed Fourier transform ^2H NMR, referred to the resonance position of external D_{12}-TMS (shown overlaid). A, D_6-diacetyldeuterohemin-cyanoferrimyoglobin in 0.1 M ionic strength phosphate buffer at pH 6.7. B, D_6-diacetyldeuterohemin-OMe in a mixture of pyridine and water, 0.75 ml and 0.25 ml, respectively, containing 4 mg KCN.

pounds are determined primarily by the four factors: the diamagnetic term, the chemical shift due to the ring current, the pseudo-contact shift, and the contact shift (5). The last two terms arise from the influence of the paramagnetic electron. Differences in environment between the protein and the free solution influence all chemical shift components.

The line width at half height in Fig. 2A for the resonance in the protein is about 26 Hz, whereas in the free solution in Fig. 2B it is 8.8 Hz. In both cases any chemical shift non-equivalence between the two methyl groups would contribute to the line width, although no direct evidence of such an effect is seen here. The line width is controlled by several factors. Since both molecules are paramagnetic Fe (III) compounds, there is a contribution to the line width from the electron-nuclear dipolar interaction and from the hyperfine coupling interaction as Solomon (6) and Bloembergen (7) have shown.

The signal broadening due to quadrupolar relaxation involves the correlation time for reorientation of the protein (τ_R) on which is impressed the rotation of the $-CD_3$ group around its axis. The signal broadening due to deuteron quadrupolar relaxation is given by (8)

$$\frac{1}{T_2} = \frac{1}{80} \quad (2\pi)^2(e^2qQ/h)^2(1+\eta^2/3)\left(9\tau_c + \frac{15\tau_c}{1+\omega_0^2\tau_c^2} + \frac{6\tau_c}{1+4\omega_0^2\tau_c^2}\right) \qquad (1)$$

which reduces to (if $4\,\omega_0^2\tau_c^2 \ll 1$, extreme narrowing, and $\eta = 0$)

$$1/T_2 = 1/T_1 = 1.5\pi^2(e^2qQ/h)^2\tau_c \qquad (2)$$

T_2 is determined from the linewidth, assuming a Lorentzian shape of the line,

$$T_2 = \frac{1}{\pi\Delta\delta} \qquad (3)$$

Here (e^2qQ/h) is the quadrupolar coupling constant, τ_c is the correlation time which involves the overall motion of the protein (τ_R) and the rotation of the label around its axes, ω_0 is the resonance frequency and $\Delta\delta$ is the linewidth at half height expressed in Hertz. η is the asymmetry parameter which can

be neglected in most cases (8). Quadrupolar coupling constants of deuterated methyl groups are often about 170 kHz (9-11).

The importance of the rotation of the $-CD_3$ group around its axis (in the case of the acetyl group there are two axes to consider) is easily shown by computing the linewidth based only on the overall tumbling of the protein (in this case $\tau_c = \tau_R$). Taking the overall correlation time, τ_R, for myoglobin from ^{13}C-NMR-relaxation measurements on the α-carbon envelope as 22 nsec (12, 13), the calculated linewidth from Equation 1 for the resonance in question would be about 1000 Hz which is in strong contrast to our actually observed linewidth of 26 Hz. Considerable rotational freedom of both of the $-CD_3$ groups within the protein clearly accounts for much of the 40-fold narrowing. This is also supported by the effective correlation coefficient, calculated by Equations 2 and 3, of 1.9×10^{-10} sec which is a factor of a hundred shorter than the overall tumbling of the protein. We want to emphasize that the τ_c value is a longer limit derived from the linewidth of the peak without making any corrections, and that no contributions to the linewidth by the paramagnetic iron are considered.

These observations illustrate the value of ^2H-NMR applied to proteins in which the deuterium label is incorporated in a relatively unconstrained structural unit. The sensitivity of the present probe to structural alterations in the myoglobin will be presented elsewhere.

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