NMR DOUBLE RESONANCE STUDY OF AZIDOFERRICYTOCHROME C

Raj K. Gupta*and Alfred G. Redfield*†
IBM Watson Laboratory, Columbia University, New York, N.Y.

Received September 4, 1970

SUMMARY

An NMR double resonance study of a mixture of ferricytochrome c and azidoferricytochrome c reveals the presence of an exchange of azide ions between the protein molecules. Irradiation of one of the several resolved methyl resonances from ferricytochrome c cross saturates a correspondingly unique resonance of azidoferricytochrome c and vice versa. The hyperfine shifted resonances of azidoferricytochrome c are thus correlated with those of ferricytochrome c and assigned to porphyrin ring methyls. "On-Off" rates for the azide ion are determined.

Azide has been widely used as an inhibitory ligand of hemoproteins and is known to block the respiratory process *in vivo*. To study the binding of azide with cytochrome c and its effect on the unpaired spin density distribution over the heme ring, we examined the NMR spectrum of cytochrome c on addition of sodium azide. A double resonance variation of the NMR method[1] was used to assign the resolved hyperfine-shifted resonances of azidoferricytochrome c to porphyrin ring methyls.

The sample consists of a 10% solution of cytochrome c in D_2O to which varying molar excess amounts of sodium azide were added (1 to 100 fold). The solutions were buffered at pD 7.0 using sodium phosphate. The NMR spectra were recorded at 100 MHz using the pulsed Fourier transform method of spectroscopy .[2]

---

*Address after September 30, 1970, IBM Thomas J. Watson Research Center, Yorktown Heights, New York.
†Address during 1970-71, Department of Biochemistry University of California, Berkeley, California.

On adding sodium azide to ferrocytochrome c, no change in the NMR spectrum was noticed, showing that azide does not bind appreciably to this protein in the reduced state.

Addition of azide to ferricytochrome c, however, alters its NMR spectrum [3]. The spectra recorded on adding varying

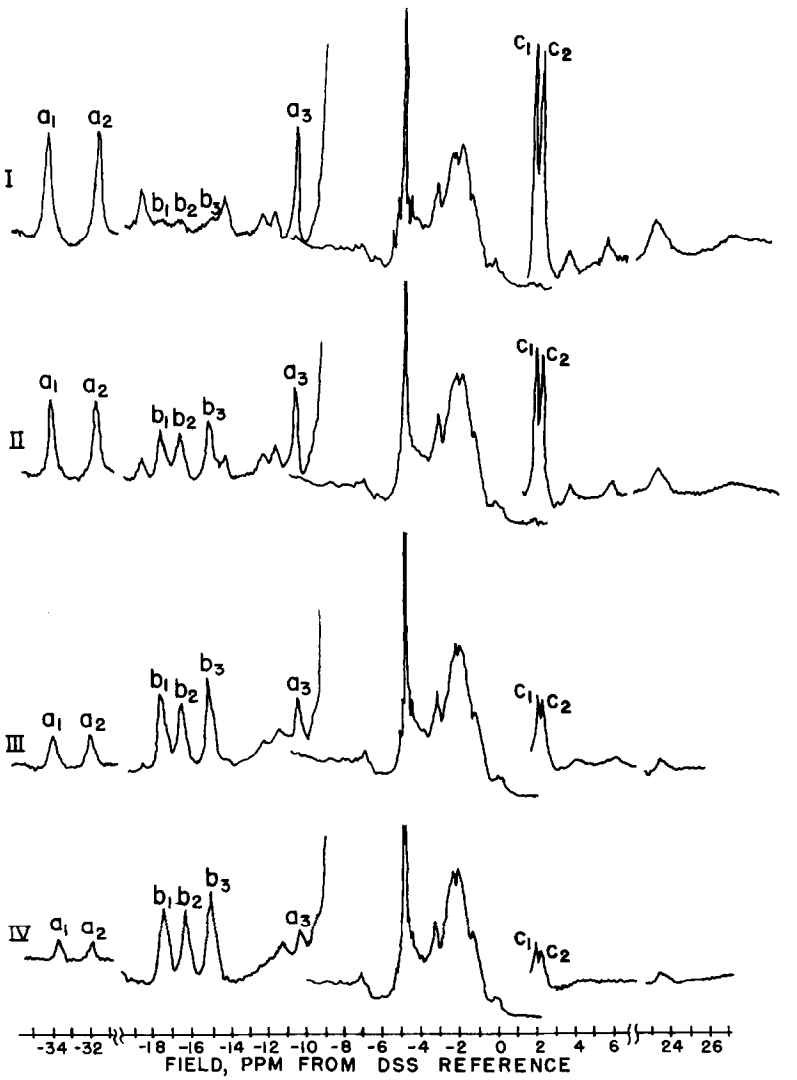

Fig. 1. NMR spectra recorded at room temperature (27°C) on adding varying excess amounts of sodium azide to ferricytochrome c. Molar ratio of ferricytochrome c and added sodium azide is: I. 1:1, II. 1:10, III. 1:50, IV. 1:100. The entire spectrum was obtained in pieces. Plotter gain was reduced by a factor of forty for the central region of the spectrum from -10 to +2 ppm.

excess amounts of sodium azide to ferricytochrome c are shown in Fig. 1. Those obtained with various non-zero concentrations of azide have the same number of lines, though they differ in their relative intensities. In particular, the relative intensities of a_1, a_2, a_3 remain the same, but they change relative to those of b_1, b_2, b_3. The a_1, a_2, a_3 lose while b_1, b_2, b_3 gain in signal intensity with increasing concentration of azide. This characteristic of the NMR spectrum where the intensity of one set of lines increases at the expense of the other is indicative of an equilibrium mixture of two species present in the solution, which in our case must be ferricytochrome c and azidoferricytochrome c. This is confirmed by the fact that all of the resolved hyperfine shifted lines in ferricytochrome c are present in this spectrum. The additional lines then must come from the complex azido-ferricytochrome c. Since even in the presence of a large excess of azide, the NMR spectrum shows the presence of appreciable amounts of uncomplexed ferricytochrome c, the binding of azide to ferricytochrome c must be weak and the azide complex tends to dissociate to parent cytochrome c and the azide ion .[4] It is generally believed that azide replaces methionine, which is known to be the weaker of the axial ligands. On reducing a solution containing the mixture of ferricytochrome c and azidoferricytochrome c with dithionite, the whole spectrum changed over to that of ferrocytochrome c within the observation time (nearly 5 minutes) as expected.

On the superposition-spectrum of ferricytochrome c and azidoferricytochrome c, we performed a double resonance experiment. This experiment consists of applying a long (0.1 sec) pulse of radio-frequency to one of the three hyper-

fine shifted (b_1, b_2, b_3) resonances of azidoferricytochrome c and observing the changes induced by the pulse. Such a pulse of rf equalizes the spin populations of the two nuclear Zeeman energy levels connected by the resonance in question by short circuiting the relaxation processes responsible for maintaining a difference in their populations at thermal equilibrium. The net result is that the signal from the irradiated resonance disappears or is reduced in intensity. The intensity of the rf pulse is fixed at a level just sufficient to saturate the resonance to which it is applied. The rest of the spectrum is then scanned by an observation pulse just after the saturating pulse to search for the changes in the spectrum induced by the latter. Since these changes are small, they are recorded conveniently by taking a difference spectrum with and without the prepulse or with the prepulse jumping in frequency between the desired resonance and some other point in the spectrum. Such a difference technique cancels any background signals that are not affected by the prepulse, and is particularly useful if the region cross-saturated happens to be in the broad overlapping part of the spectrum. A difference spectrum recorded with double irradiation prepulse alternating between the hyperfine shifted resonances b_1 and b_3 of azidoferricytochrome c is shown in Fig. 2. The only frequencies where the difference spectrum is non-vanishing other than the ones directly irradiated are the positions of the cross saturated resonances. These have been identified earlier by us as porphyrin ring methyls using the same double resonance technique to correlate the resonances of ferri and ferrocytochrome c [1]. In this way we now identify the hyperfine shifted resonances of azidoferricytochrome c at -14.8, -16.1, -17.3 ppm with those of ferricytochrome c at

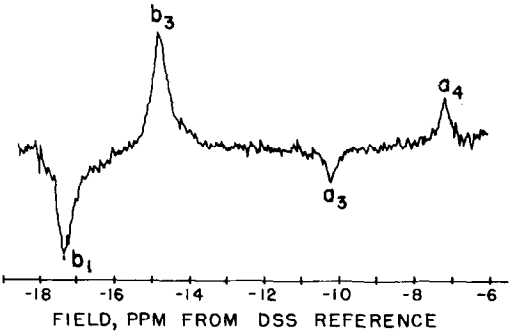

Fig. 2. A difference spectrum recorded with double irradiation pulse jumping between b_1 and b_3. The a_3 and a_4 are the positions of cross saturated resonances.

-7.2, -34.0, -10.3 ppm, respectively. The correlation also assigns them to porphyrin ring methyls. It is possible in this case to do a reverse experiment where one saturates the ferricytochrome c resonances and observes changes induced in the azidoferricytochrome c spectrum. We tried unsuccessfully to locate the corresponding positions of the ferricytochrome c methyl at -31.4 ppm and the methionine at 23.4 ppm in the azidoferricytochrome c spectrum. This failure may be due to the excessive broadening of these resonances by hyperfine interaction leading to difficulties in their detection.

A careful look through the spectra (Fig. 1) reveals that besides changes in relative intensities with increasing concentrations of azide, there are observable changes in the resonance positions of some of the lines too. In particular, the separation between the low field methyl peakes of ferricytochrome c, a_1 and a_2, decreases by about 1 ppm in going from equimolar to 100 fold molar excess amounts of azide. Resonance a_1 tends to shift upfield while a_2 downfield with increasing azide concentration. The unpaired spin density distribution thus seems to be sensitive to solvent perturba-

tions. It is, however, possible that azide has more than one binding sites in the molecule and that the observed shift arises from a fast exchange between free cytochrome and the complexed cytochrome c with azide bound at the second site. Fast exchange between complexes with at least one azide liganded to iron could account for any observed changes in the azidoferricytochrome c spectrum with increasing azide concentration. The azidoferricytochrome c resonances corresponding to c_1 and c_2 of ferricytochrome seem to occur at -0.5 and 1.0 ppm. The uncertainty in establishing their positions lies in the small separation between the corresponding lines of the two forms in this case.

It is interesting to note from a comparison of the hyperfine shifted regions of ferricytochrome c and azidoferricytochrome c spectra that replacing methionine by azide as the axial ligand coordinated to the face of the heme alters the pattern and symmetry of the unpaired spin density distribution over the heme ring drastically.

The very success of the double resonance experiment also establishes that each molecule of the protein alternates between ferricytochrome c and azidoferricytochrome c with a mean life-time in either state of the order of the spin lattice relaxation time in that state. We can measure this life time and therefore the association and dissociation constants for azide ion precisely.

The basis for the measurement is as follows: Let us assume that the two states (ferri and azidoferricytochrome c) are denoted by α and β. τ_α, τ_β and M_z^α, M_z^β are their life times and magnetizations, respectively. M_o^α and M_o^β are the thermal equilibrium values of the magnetizations. The time

dependence of magnetization in the α state in the presence of a saturating prepulse at the β state is governed by the equation[5]:

$$\frac{dM_z^\alpha}{dt} = \frac{M_o^\alpha - M_z^\alpha}{T_1^\alpha} - \frac{M_z^\alpha}{\tau_\alpha} + \frac{M_z^\beta}{\tau_\beta}$$

and a similar equation for M_z^β. It may be noted that the ratio of life-times $\tau_\alpha/\tau_\beta = M_o^\alpha/M_o^\beta$. T_1^α is the spin lattice relaxation time in the α state in the absence of exchange. When steady state is attained in the presence of rf field at β state resonance,

$$\frac{dM_z^\alpha}{dt} = 0 \qquad M_z^\beta = 0$$

yielding

$$\tau_\alpha = T_1^\alpha \frac{M_z^\alpha/M_o^\alpha - M_z^\beta/M_o^\beta}{1 - M_z^\alpha/M_o^\alpha}$$

M_z^α/M_o^α, M_z^β/M_o^β can be determined experimentally as the ratios of the signals in the presence and absence of the rf prepulse at least for the well-resolved hyperfine shifted lines. Knowing T_1^α, the spin lattice relaxation time of the protons in question in ferricytochrome c in the absence of azide complex, one can measure τ_α precisely. τ_β is then easily obtained as $\tau_\alpha M_o^\beta/M_o^\alpha$. We measured τ_α employing the resonance a_1 of ferricytochrome c which cross saturates with b_2. Spin lattice relaxation time (T_1^α) for this resonance (a_1) in the α-state (ferricytochrome c) was measured by employing a 180^o-90^o pulse sequence[6]. A value of 30 ms was obtained for T_1^α. The values of τ_α obtained in this way are 350 ms and 70 ms at azide concentrations 150 mM and 750 mM, respectively. It is seen that the life-time of ferricytochrome c state is

approximately inversely proportional to azide concentration, as might be expected for a bimolecular reaction. τ_β was not measured directly since in the solutions employed there was always a certain amount of uncomplexed ferricytochrome c present which made the measurement of T_1^β difficult. τ_β was, however, calculated by measuring the ratio M_o^β/M_o^α. The values obtained in this way are 230 ms and 140 ms for solutions with 150 mM and 750 mM concentrations of azide, respectively. The variation in τ_β implied was confirmed by studying the magnitudes of cross saturation effect in the two cases. Although it is a bit puzzling to note τ_β change with the azide concentration, the effect could easily be understood in terms of weakening of Fe^{3+}-azide bond due to solvent perturbations. The alternative explanation involving a second binding site for azide, invoked earlier in this paper, would require a decrease in the azide binding constant of the first site in the presence of azide bound at the second site. Both the explanations appear equally feasible at present and further work would be required to establish either.

The chemical equation representing the equilibrium in our case is:

$$\text{Cytochrome c} + \text{Azide} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Azidocytochrome c}$$

k_1 is the rate constant for the forward reaction or association. k_{-1} is the rate constant for the reverse reaction or dissociation. The life times τ_α and τ_β are related to k_1 and k_{-1} as follows:

$$\tau_\alpha = \frac{1}{k_1[\text{Azide}]} \quad \text{and} \quad \tau_\beta = \frac{1}{k_{-1}}$$

The square brackets denote the molar concentration of the

species enclosed. The so-called binding constant is easily obtained as $K_{eq} = k_1/k_{-1} = \tau_\beta/\tau_\alpha[\text{Azide}] = M_o^\beta/M_o^\alpha[\text{Azide}]$. The values of k_1 and k_{-1} obtained in this way are 20/mol. sec. and 4/sec., respectively and the binding constant is 5/mol. at low azide concentrations.

REFERENCES

1. Gupta, R. K. and Redfield, A. G., Science (In Press)
2. We use a JEOLCO 100 MHz spectrometer converted to pulsed operation. A detailed report describing this instrument is available from us on request.
3. Wüthrich, K., unpublished.
4. Horecker, B. L. and Stannard, J. N., J. Biol. Chem. 172, 589 (1948).
5. Johnson, C. S., Advan. Magnetic Resonance 1,33 (1965).
6. Vold, R. L., Waugh, J. S., Klein, M. P. and Phelps, D. E., J. Chem. Phys., 48, 3831 (1968).