Biochimica et Biophysica Acta, 292 (1973) 502–508

BBA Report

BBA 41229

Nuclear magnetic resonance study of the interaction of cytochrome *c* with cytochrome *c* peroxidase

RAJ K. GUPTA and TAKASHI YONETANI

Laboratory of Chemical Physics, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Md. 20014 and Department of Biophysics and Physical Biochemistry, Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa. 19104 (U.S.A.)

(Received January 2nd, 1973)

SUMMARY

The hyperfine shifted resonances in the nuclear magnetic resonance (NMR) spectrur of cytochrome *c* broaden upon addition of cytochrome *c* peroxidase in a manner which indicates 1:1 reversible complex formation between cytochrome *c* and cytochrome *c* peroxidase. Since, in the presence of excess cytochrome *c*, we see a time-averaged NMR spectrun and not a simple superposition of two distinct spectra from free and complexed cytochrome *c*, the exchange between the two forms is fast on the NMR time scale, yielding for the off-rate of the complex a lower limit of 200/s. (The on-rate is several order of magnitude greater than the off-rate.)The observed line widths of methyl resonances are, consistent with theory, dependent on the rotational correlation time of the whole macromolecule/macromolecular complex. The widths of the heme ring methyls of cytochrome *c* in cytochrome *c*–peroxidase complex are not sensitive to the electronic spin state changes in the cytochrome *c* peroxidase heme iron on addition of cyanide or fluoride showing that the heme groups of cytochrome *c* and cytochrome *c* peroxidase are sufficiently far from each other. The unpaired electron spin density distribution over the heme ring of cytochrom *c* in cytochrome *c*–peroxidase complex is slightly altered with respect to free cytochrome *c* as indicated by observed small changes in the positions of the ring methyl resonances. This could arise from a small conformational change in cytochrome *c* on complexing with cytochrome *c* peroxidase.

Cytochrome *c* peroxidase is a mitochondrial enzyme responsible for the peroxidation of ferrocytochrome *c* in yeast. The mode of interaction of this enzyme with cytochrome

c is incompletely understood at present. Formation of reversible complexes between cytochrome *c* peroxidase and both reduced and oxidized cytochrome *c* has often been postulated[1,2] However, no direct spectroscopic evidence for the existence of such complexes is yet available. The mechanism of electron transfer in peroxidation of cytochrome *c* by cytochrome *c* peroxidase is of interest in view of its analogy to the oxidation of cytochrome *c* by mammalian cytochrome *c* oxidase. In this paper, we present nuclear magnetic resonance (NMR) data which, in addition to providing direct evidence for reversible 1:1 complex formation between cytochrome *c* and cytochrome *c* peroxidase, indicate lack of heme–heme interaction in the complex.

The NMR spectrum of horse heart cytochrome *c* in the oxidized state is well characterized by the presence of two low field methyl resonances at −35 and −32 ppm, a broad high field resonance at +24 ppm (see Fig. 1a) and two other relatively sharp high field methyls at +2.4 and +2.7 ppm (at room temperature (25 °C) relative to the standard 2,2-dimethyl-2-silopentane 5-sulfonate reference)[3]. The high field broad resonance has been

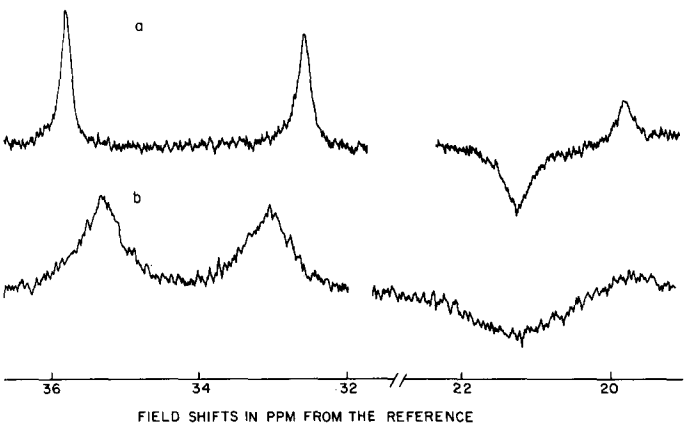

Fig. 1. The hyperfine-shifted low field porphyrin ring methyl resonances and the inverted image of the broad high field absorption due to the methionine methyl group (appearing 10 KHz downfield from the actual resonance position) of cytochrome *c* recorded at 220 MHz (20 °C) using 2,2-dimethyl-2-silopentane 5-sulfonate as the reference. (a) In free protein; (b) in the presence of equimolar amounts of cytochrome *c* peroxidase.

assigned to the methyl group of the methionine −80 which is coordinated to the heme[3,4]. The lowest field resonance at −35 ppm has been assigned to the heme ring methyl buried inside the protein having a carboxylic acid side chain next to it. This is the same methyl which has the tryptophan-59 close to it in the reduced state[4]. The other resonance at −32 ppm arises from the ring methyl on the diagonally opposite pyrole ring, which is on the exposed edge of the heme[4,5]. The latter assignment is based on the consideration of two-fold symmetry for the iron *d* orbital containing the unpaired electron about the axis normal to the heme[4]. Assignments of the two upfield resonances at 2.4

and 2.7 ppm are somewhat uncertain. They could be arising from porphyrin side chain methyls or methyls on the polypeptide chain[4]

Cytochrome *c* used in this study was the purified commercial preparation obtained from Sigma Chemical Company (horse, type VI). Cytochrome *c* peroxidase (mol. wt 36 000) was prepared by the method of Yonetani and Ray[6].

The mixture of cytochrome *c* and cytochrome *c* peroxidase was electrodialysed to remove small ions which might interfere with our experiments. The pH of the solution varied from 6.5 to 8 depending on the relative amounts of the peroxidase and cytochrome *c* used. The hyperfine shifted NMR spectrum of cytochrome *c* is invariant in this pH range[7]. NMR spectra were recorded at 100 as well as 220 MHz at room temperature (approx. 20 °C). Our experimental results are summarized below.

(1) For free cytochrome *c*, we measure a line width (full width at half height) of about 30 Hz for the low field methyls, 100 Hz for the high field broad resonance and 20 Hz for the other two high field resonances. The low field methyls have a spin-lattice relaxation time of 30–35 ms and the broad high field of 2.5 ± 0.5 ms (ref. 4). The methionine methyl resonance in the reduced state has a width of about 16 Hz.

(2) In the presence of equimolar amounts of cytochrome *c* peroxidase and cytochrome *c*, there is appreciable broadening of all ferricytochrome *c* resonances. Both low field resonances broaden to 100 Hz and the broad high field resonance to around 250 Hz (see Fig. 1). The two high field resonances at 2.4 and 2.7 ppm overlap under these conditions making width measurements difficult. However, discounting the effect of the overlap, we estimate the widths of these resonances to be 80 Hz in the presence of equimolar amounts of cytochrome *c* peroxidase.

(3) There is an observable shift (nearly 1/2 ppm) in the positions of the low field absorptions, although the resonance frequency of the broad high field absorption does not seem to be affected significantly by the presence of cytochrome *c* peroxidase. The resonance at −35 ppm moves in closer to the broad overlapping part of the protein spectrum and the one at −32 ppm moves away from it. The separation between the low field peaks is 2 ppm in the presence of equimolar amounts of cytochrome *c* peroxidase compared with a separation of 3 ppm in free cytochrome *c*.

(4) On adding increasing amounts of cytochrome *c* peroxidase to a cytochrome *c* solution, the separation between the low field resonances changes progressively in a linear manner until a minimum separation is reached at 1:1 ratio of cytochrome *c* and peroxidase concentrations. This is shown in Fig. 2. Addition of more cytochrome *c* peroxidase (above the concentration of cytochrome *c*) does not produce any further shift of the spectrum. The line widths also reach a maximum at equimolar concentrations of the peroxidase and cytochrome *c*. Addition of more cytochrome *c* peroxidase does not produce any further broadening of the spectrum.

(5) Complexing the cytochrome *c* peroxidase heme with cyanide and fluoride which alter its electronic state (Yonetani, T., Ehrenberg, A. and Schleyer, H., unpublished results) does not alter the widths of cytochrome *c* resonances in an equimolar mixture of cytochrome *c* and peroxidase.

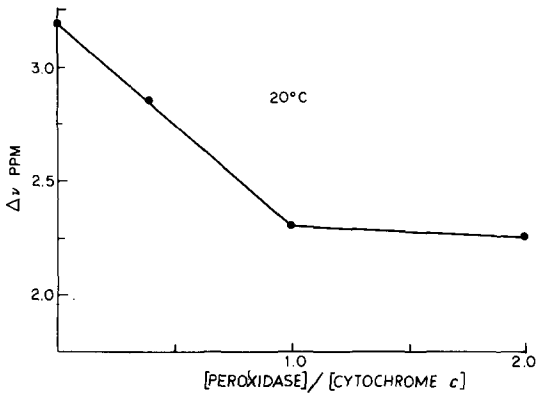

Fig. 2. The separation of the two hyperfine shifted low field porphyrin ring methyl resonances ($\Delta\nu$) of cytochrome *c* plotted as a function of the ratio of cytochrome *c* peroxidase and cytochrome *c* concentrations.

The observed line width of a low field methyl is a sum of proton–proton dipolar as well as electron–proton dipolar and scalar contributions. Since, in our case, electron–proton interaction is expected to contribute equally to both T_1 and T_2, we expect a 10-Hz paramagnetic contribution to the line widths of the low field methyls. Thus the proton–proton dipolar contribution to the line widths of the ring methyl resonances is estimated to be around 20 Hz, which also agrees fairly well with the measured value of about 16 Hz for the width of methionine methyl in the reduced state. The measured 20-Hz width of the two high field methyls at 2.4 and 2.7 ppm (not shown in Fig. 1) can be understood, in the light of the above, entirely in terms of proton–proton dipolar interactions.

The progressive broadening with increasing concentration of cytochrome *c* peroxidase observed must be interpreted in terms of reversible complex formation between cytochrome *c* and peroxidase. There could be a broadening effect just because of the presence of slowly relaxing high spin iron–protein in solution, but to give the observed magnitude of broadening, the concentration of cytochrome *c* peroxidase will have to be much more than that used in the present experiments. The broadening observed from this kind of a situation is expected to keep increasing with increasing concentration of cytochrome *c* peroxidase and not reach a maximum at 1:1 ratio of peroxidase and cytochrome *c* concentrations. In addition, shifts in the spectrum other than susceptibility shifts could not be expected. Equal broadening for the two low field methyls further confirms that unbound cytochrome *c* peroxidase which will be expected to give a differential broadening effect on these two methyls could not be playing a role in the observed broadening.

Cytochrome *c* peroxidase has a mol. wt of about 36 000. When it binds to cytochrome *c*, the cytochrome *c*–peroxidase complex will have a mol. wt of about 48 700. This is roughly 4 times the mol. wt of cytochrome *c* (12 700). Thus the cyto-

chrome *c*–peroxidase complex will tumble much more slowly in solution than free cytochrome *c*. The tumbling correlation time (τ_c) is inversely proportional to the size or volume of the molecule. Assuming the mean density of matter being constant in cytochrome *c* molecule and cytochrome *c*–peroxidase complex, one would expect the tumbling correlation time of the complex to be four times more than that of free cytochrome *c* in solution. This change in some way must be affecting the line widths. It is well known that increasing the rotational correlation time broadens the resonances. Usually the shortest correlation time, corresponding to the fastest rotational motion in a molecule, is the one that determines the proton–proton dipolar contribution to the line widths. In our case, the rotational motion of the methyl group around the carbon–carbon bond axis is expected to be the fastest motion. However, this motion is not isotropic as is expected theoretically, and is borne out experimentally from the observed broadening. When the fast motion is anisotropic, the final averaging of the proton–proton dipolar interaction must be done by the slower tumbling motion of the macromolecule (or macromolecular complex) as a whole. The line width for two protons interacting with each other is, in such a case, given by (Redfield, A.G., unpublished);

$$\Delta\nu_{obs} = \left(\frac{3\cos^2\theta - 1}{2}\right)^2 \Delta\nu_o$$

where $\Delta\nu_o$ is the line width in the absence of the fast motion which is proportional to τ_c where τ_c is the tumbling correlation time for the whole molecule. θ is the angle between the axis of rotation and the proton–proton vector. Our observation that the line widths increase with increasing complexing indicates that this is indeed the case and that the tumbling correlation time of the whole molecule does affect the line widths. Since the correlation time for the electron–proton hyperfine interaction, which represents approximately 10 Hz contribution to the line widths of the low field methyls, is independent of the tumbling correlation time of the whole molecule, being determined by the electronic relaxation time which is several orders of magnitude shorter than the former, one expects the line widths of the low field ring methyls in the cytochrome *c*–peroxidase complex to be (20 X 4 + 10) Hz in good agreement with the experimental value of about 100 Hz. The observed broadening of the two upfield methyl resonances at +2.4 and +2.7 ppm by a factor of 4 in going from free cytochrome *c* to cytochrome *c*–peroxidase complex lends further support to this view. For the broad high field methyl resonance, one would expect a line width of about 200 Hz which is somewhat less than the experimentally observed value of 250 Hz. The small discrepancy may not be serious since there is a problem in measuring linear widths accurately for this broad resonance. Also there may be a methionine methylene proton buried under this broad methyl resonance[3] which complicates the situation. It is also possible that the electronic relaxation is not sufficient to average out the dipolar interaction completely, the magnetic moment of the electron being anisotropic (the inverse of the anisotropy expressed in frequency units being comparable to the relaxation rate of the electron). The final averaging of the

electron–proton dipolar interaction in this case may have to be done by the slower tumbling motion. A theoretical treatment of nuclear relaxations in this complex situation is not yet available in the literature. A small conformational change in cytochrome *c* which will push the methionine methyl group closer to iron on binding with cytochrome *c* peroxidase could also account for the observed line width in the complexed state.

The fact that the resonances shift upon addition of the peroxidase shows that the unpaired electron spin density distribution over the heme in the cytochrome *c*–peroxidase complex is not the same as that in free cytochrome *c*. The spin density on the outer ring methyl is increased and that on the inner one decreased by about 2%. This could merely be the effect of the net negative charge of cytochrome *c* peroxidase which will repel the unpaired electron cloud in the complex away from it. It is also possible that the binding of the peroxidase to cytochrome *c* produces a conformational change which alters the resonance positions. In principle, it should be possible to decide between these two alternative explanations by studying the effect of added anions on the shift. A shift arising due to the proximity of the negative charge of cytochrome *c* peroxidase molecule should disappear on screening the charge with small ions. Unfortunately cytochrome *c*–peroxidase complex dissociates at the high ionic strengths needed for effective screening, perhaps because the forces holding cytochrome *c* and the peroxidase together in a complexed state are of electrostatic origin.

Since we find that all cytochrome *c* is complexed when an equal amount of the peroxidase is present, the binding of cytochrome *c* peroxidase to cytochrome *c* must be strong (high equilibrium constant) and 1:1. When excess cytochrome *c* is present, we observe a time-averaged NMR spectrum of free and complexed cytochrome *c* and not a simple superposition of two distinct spectra. This indicates that the on–off rates for the cytochrome *c*–peroxidase complex must be much greater than the inverse of the frequency separations between the complexed and uncomplexed state (nearly 200 Hz at 220 MHz NMR frequency). Since the equilibrium constant is very high, the on-rate for complexation must be several orders of magnitude greater than the off-rate. From our data, then, we could set a lower limit of 200/s on the off-rate.

It is of great interest to find out if the electron from ferrocytochrome *c* transfers directly from its heme to the peroxidase heme or if the transfer occurs *via* the protein moiety. The separation between the cytochrome *c* and peroxidase hemes in the cytochrome *c*–peroxidase complex may throw some light on this aspect. Since the heme iron of the peroxidase is slow relaxing, it is expected to broaden significantly the resonances of protons close to it. If part of the broadening we observe were coming from this effect, we might have been able to estimate the heme–heme distance in the cytochrome *c*–peroxidase complex. Unfortunately this does not seem to be the case for the following reasons: (1) Equal contribution to the broadening of the two ring methyls well assigned to groups on the diagonally opposite ends of the cytochrome *c* heme is very unlikely, *i.e.* it is improbable that the peroxidase heme iron will occupy a position symmetrical with respect to these two ring methyls in the cytochrome *c*–peroxidase complex.(2) The widths of the resonances in the cytochrome *c*–peroxidase complex are not sensitive to

changes in the electronic structure of the peroxidase heme (and consequently also electronic relaxation time) on complexing with cyanide and fluoride. These observations must mean that the cytochrome *c* and the peroxidase hemes in cytochrome *c*–peroxidase complex are sufficiently far from each other (>25 Å, assuming electronic relaxation time of the high spin heme iron of peroxidase approx. 10^{-10} s) and do not interact appreciably. This suggests the possibility of electron transfer *via* the polypeptide chain.

It is a pleasure to acknowledge useful discussions with Prof. E. Margoliash, J. Leigh and A.G. Redfield. One of us (R.K.G.) wishes to express gratefulness to Prof. Edwin D. Becker for providing an opportunity to pursue cytochrome *c* studies in his laboratory at N.I.H.

REFERENCES

1 Nicholls, P. and Mochan, E. (1971) *Biochem. J.* 121 55–67
2 Mochan, E. and Nicholls, P. (1971) *Biochem. J.* 121, 69–82
3 Wüthrich, K. (1969) *Proc. Natl. Acad. Sci. U.S.* 63, 1071–1078
4 Redfield, A.G. and Gupta, R.K. (1971) *Proc. Cold Spring Harbor Symp. Quant. Biol.* 36, 405–411
5 Dickerson, R.E., Takano, T., Eisenberg, D., Kallai, O.B., Samson, L., Cooper, A. and Margoliash, E. (1971) *J. Biol. Chem.* 246, 1511–1535
6 Yonetani, T. and Ray, G.S. (1965) *J. Biol. Chem.* 240, 4503–4508
7 Gupta, R.K. and Koenig, S.H. (1971) *Biochem. Biophys. Res. Commun.* 45, 1134–1143