Determination of Formal Potentials of Multihemoprotein, Cytochrome c_3 by ^{1}H Nuclear Magnetic Resonance

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Macroscopic redox potentials of tetrahemoprotein, cytochrome c_3 from *D.* vulgaris, Miyazaki strain, were derived from 400 MHz ^{1}H NMR measurements. The formal potential, $E_1^{\circ\prime}$, was determined to be -0.245 ± 0.005 V *vs.* NHE compared with a value of -0.240 V obtained electrochemically. The distributions of the intermediate states from the NMR spectra during a redox titration are in good agreement with those previously determined by electrochemistry.

Cytochrome c_3 (cyt. c_3) has four hemes within a single polypeptide chain and functions as an electron carrier in the sulfate reducing bacteria, *Desulfovibrio vulgaris.* The oxidoreduction of cyt. c_3 is regulated by the catalytic action of hydrogenase, an iron-sulfur enzyme. The physicochemical properties of the cyt. c_3 have been well studied by Mössbauer spectroscopy,[1] photoelectron spectroscopy,[2] electrical conductivity,[3] electrochemical methods,[4,5] ESR,[6] and NMR.[7] These studies have revealed that this protein has unusual properties such as a very low ionization potential, high electrical conductivity, and a rapid and reversible redox reaction owing to its multiheme structure. It also has been suggested that the redox potentials of the hemes are connected with these properties.

The macroscopic redox potentials of this multihemeprotein derived from 400 MHz ^{1}H NMR measurements are reported. These potentials have previously been obtained by the simulation of the differential pulse voltammetric response during redox titrations. The redox potential, E, of cyt. c_3 is defined by

$$E = E_{i+1}^{\circ\prime} + (RT/F)\ln[f_i]/[f_{i+1}] \quad (1)$$

where $E_{i+1}^{\circ\prime}$ is the macroscopic formal potential for the $f_i \rightleftharpoons f_{i+1}$ equilibrium and where f_i is the ith oxidation state as shown in Fig. 1. It should be noted in this figure that there is an unity (one electron) difference for each oxidation state. There are five states corresponding to n=0, 1, 2, 3, and 4 electrons (designated by f_0 to f_4) while states f_1—f_3 can have multiplicities arising from the multicomponent electron sites.

Experimental

D. vulgaris cyt. c_3 was purified by a method previously reported.[8] The purity index $[A_{552}(Fe^{2+})/A_{280}(Fe^{3+})]$ was 3.0. The protein was lyophilized twice from 99.95% D_2O. Hydrogenase was also purified according to the method described by Yagi *et al.*[9] The lyophilized powder of cyt. c_3 and a trace amount of deuterized hydrogenase were dissolved into the deuterized phosphate buffer solution (pD=7.0), giving a final cyt. c_3 concentration of 5 mM (1 M=1 mol m^{-3}). The pD values were measured by a pH meter. The NMR spectra were recorded and analyzed using a JEOL GX 400 spectrometer. After measurements on the ferricytochrome c_3, it was reduced to the ferro-form by hydrogen. The samples of various intermediate oxidation states were prepared by the reoxidation of the fully reduced sample with air. All measurements were conducted at (25±0.5) °C, and the chemical shifts were quoted in part per million, down field from sodium 3-(trimethylsilyl) propionate-2,2,3,3-d_4(TSS).

The optical absorption spectra of the *ca.* 0.1 mM cyt. c_3 of an intermediate oxidation state were measured in the NMR tube before and after NMR measurement. We confirmed that there were no significant differences in the optical spectra taken before and after NMR measurement. In the case of the 0.1 mM cyt. c_3 solution, the number of accumulation of free induction decay was 10,000 in order to increase the signal to noise ratio. However, this acquisition number was still not large enough for a good signal to noise ratio and a smoothing technique was used to analyze the NMR lineshape.

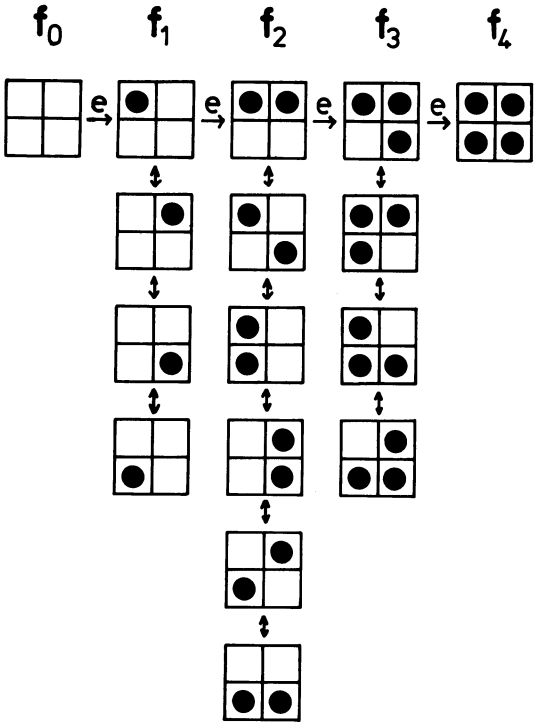

Fig. 1. Microscopic oxidation states of cyt. c_3. Solid circles indicate the reduced sites. f_0—f_4 indicate macroscopic oxidation states.

Results and Discussion

Figure 2 shows the NMR spectra in the lower field region for a redox titration of cyt. c_3. The sharp singlet peaks (A—P) with three-proton intensity in the region of 31—8 ppm were assigned to the heme methyl resonances of the f_0 state.[7,10] As the amount of reduction is incrementally increased, (spectra # 1—9 in Fig. 2) new peaks are seen to appear at the expense of the f_0 intensity. The height of all f_0 peaks decreased simultaneously. This is due to the fact that the intramolecular electron-exchange rate of cyt. c_3 is so rapid on the NMR time scale that the microscopic states in each intermediate oxidation state, f_1, f_2, and f_3, are indistinguishable, so it is the average fraction of each intermediate state that is determined by the NMR. From the intensity of five well-isolated peaks (A,B,C,F,I), the reduction ratio of the f_0 state was obtained. The new peaks that appeared at 27.7, 20.0, 19.55, and 18.0 ppm were assigned to f_1, and those at 15.36, 14.1, and 13.8 ppm were assigned to f_2. The assignment was done by the saturation transfer method.[7]

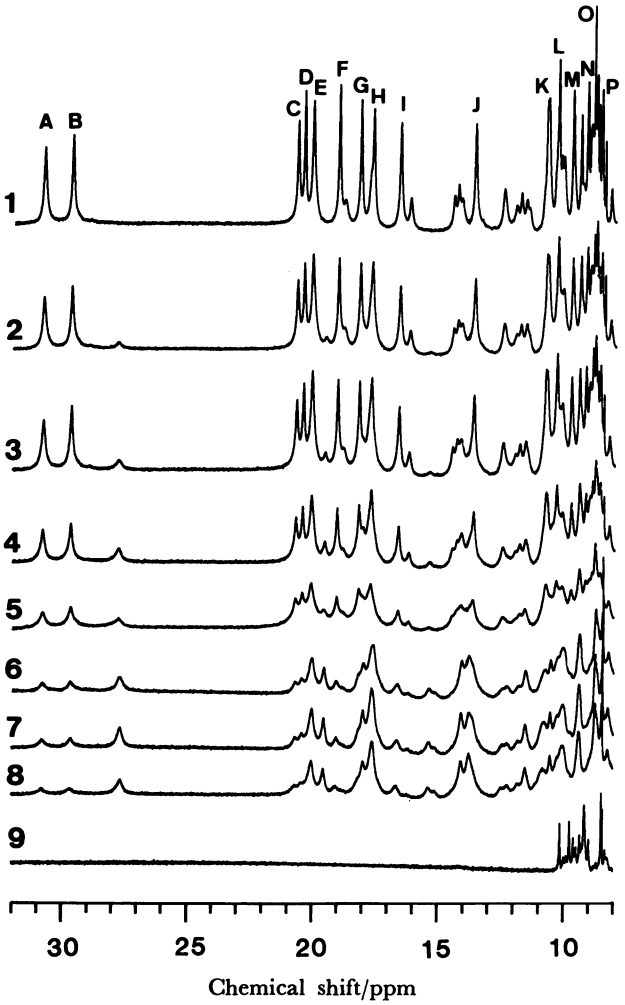

Fig. 2. NMR spectra for a redox titration of cyt. c_3 recorded on a 400 MHz NMR: spectrum (1) is fully oxidized cytochrome c_3, (2) f_0=0.94, (3) =0.93, (4)= 0.665, (5)=0.42, (6)=0.22, (7)=0.16, (8)=0.10, and (9) is fully reduced cyt. c_3.

From the peak area of each of the oxidation states, one can calculate the fractions of the f_0, f_1, and f_2 states. The macroscopic formal potential, $E_1^{\circ\prime}$ for the equilibrium between f_0 and f_1, can be determined from the ratio of the f_0 state to the f_1 state and was estimated to be (−0.245±0.005) V, which is in good agreement with that determined by the electrochemical method, −0.239 V, in D_2O. Similarly, one can calculate the macroscopic formal potentials of the intermediate states from the fraction of each intermediate state.

The macroscopic formal potentials of cyt. c_3 from *D. vulgaris* determined by electrochemical method in both H_2O and D_2O are given in Table 1. On the basis of the macroscopic formal potentials given in Table 1, we can calculate the relative concentrations of the five redox states defined in Fig. 1 as a function of the electrochemical potential as shown in Fig. 3. In the upper part of this figure, arrows designate the points at which UV and redox titration measurements were conducted. At the bottom of the figure, the four macroscopic formal potentials are shown. The broken line indicates the average fraction of the ferri-heme in cyt. c_3, which is observed by optical absorption spectroscopy. The ratios, f_1/f_0 and f_2/f_0, calculated from

Table 1. Macroscopic formal potentials of cytochrome c_3, *D. vulgaris*, Miyazaki strain (V *vs.* NHE)

	in D_2O (pD=7.1)	in H_2O (pH=7.1)
$E_1^{\circ\prime}$	−0.239	−0.240
$E_2^{\circ\prime}$	−0.293	−0.297
$E_3^{\circ\prime}$	−0.310	−0.315
$E_4^{\circ\prime}$	−0.352	−0.357

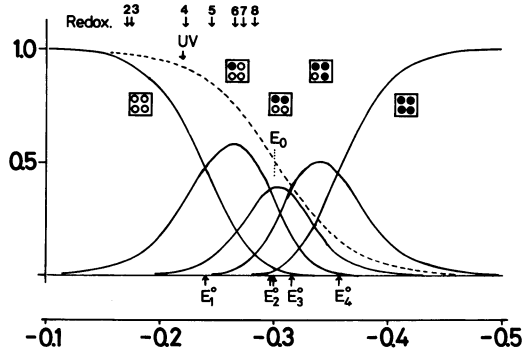

Fig. 3. Population of five macroscopic oxidation states calculated from the electrochemical data as a function of electrochemical potentials. Arrows on the top of the figure designate the intermediate oxidation states (2) to (8) shown in Fig. 2.

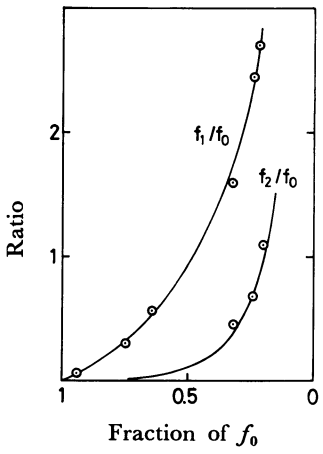

Fig. 4. The f_1/f_0 and f_2/f_0, as a function of the fraction of the f_0 state. Open circles are calculated from NMR data; solid lines, from electrochemical data.

the NMR data (open circles) and those from the data shown in Fig. 3 (solid lines) are plotted in Fig. 4 as a function of the fraction of the f_0 state. The NMR data fit well with the curve calculated from electrochemical methods. These results strongly suggest that the four macroscopic formal potentials of the cyt. c_3 are a unique set of formal potentials. The well resolved spectra in the present study gave the rigid foundation to assign the absorption lines, many of which were compatible with the early work.[7] Based upon their poorly resolved spectra.[11] Santos *et al.* derived four redox potentials of *D. gigas* which were quite different from the present study.

With respect to the reaction scheme between the microscopic states as shown in Fig. 1, we should emphasize that the intramolecular and intermolecular electron exchange rates and the microscopic formal potentials of cyt. c_3 must be studied. Further NMR studies are now in progress.

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