Volume 308, number 3, 264-266 FEBS 11445 August 1992

Non-equivalent natures of the coordinated imidazole rings of cytochrome c_3 from *D. vulgaris* Miyazaki F as studied by ^{1}H NMR

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Received 20 May 1992; revised version received 28 June 1992

All of the C2 proton signals of the coordinated histidine residues in the ^{1}H NMR spectrum of cytochrome c_3 from *D. vulgaris* Miyazaki F were assigned by specific deuteration. They appeared at extremely high fields and scattered in a wide range from −4 to −22 ppm. This clearly shows that the chemical properties of the imidazole groups are quite different from one another. The extremely high-field shift of the C2 signal indicates that some of them must carry the imidazolate-like nature to some extent. This might be responsible for the extremely low redox potentials of the four hemes. On changing temperature, most of them showed Curie-type change. All of the C2 signals showed a small p^2H dependence in the range of p^2H 4.8-10.0.

Cytochrome c_3; Tetraheme protein; Coordinated histidyl imidazole; Redox potential; ^{1}H NMR

1. INTRODUCTION

Cytochrome c_3 is a unique class of heme protein which contains four hemes in a single polypeptide [1]. Crystal structures of cytochrome c_3 from *Desulfovibrio desulfuricans* Norway and *Desulfovibrio vulgaris* Miyazaki F (*Dv*MF) have been reported [2,3]. All of the 5th and 6th ligands of the four hemes are histidyl imidazoles. One of the remarkable features of this protein is the extremely low redox potential of the four hemes in comparison with other *c*-type cytochromes. The formal potentials of each heme (the microscopic redox potentials) of *Dv*MF cytochrome c_3 were estimated by the use of NMR [4]. The values of 32 microscopic redox potentials were in the range from −265 to −370 mV vs. NHE (normal hydrogen electrode). The redox potentials were assigned to the specific hemes in the crystal structure [5]. The crystal structure, however, did not give a clue to the extremely low redox potentials. We have carried out the assignment of the C2 (ε1) proton signals of ^{1}H NMR spectrum of *Dv*MF cytochrome c_3 in this work, which suggested the role of the imidazole rings in realizing the low redox potentials.

2. MATERIALS AND METHODS

D. vulgaris Miyazaki F was cultured in medium C [1] and in a minimal medium [6] to obtain non-deuterated and deuterated cytochrome c_3, respectively. In the latter case, the C2 (ε1) proton of the histidyl imidazole of cytochrome c_3 was specifically deuterated by replacing L-histidine of the minimal medium with deuterated L-histidine. The C2 position of L-histidine was deuterated by incubating it in ^2H_2O (99.75% Showa Denko) at p^2H 8.2 and 80°C for 48 h. Cytochrome c_3 was purified according to the reported procedure [6]. The purity was checked by the purity index (A_{553}(red)/A_{280}(ox)) and SDS-PAGE. 400 MHz ^{1}H NMR spectra were measured with a Bruker AM-400 NMR spectrometer. Chemical shifts are presented in parts per million (ppm) to the internal standard 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The p^2H values reported in this paper are pH meter readings uncorrected for isotope effects.

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3. RESULTS

There are nine histidine residues in the cytochrome c_3 (cyt c_3) from *D. vulgaris* Miyazaki F (*Dv*MF), eight of which are ligands of the four hemes. 400 MHz ^{1}H NMR spectra of non-deuterated and deuterated *Dv*MF ferricytochrome c_3 are presented in Fig. 1. In the spectrum of non-deuterated cytochrome c_3 (Fig. 1A), eight extremely broad signals were observed in the region higher than 0 ppm, which disappeared on the deuteration of the C2 position of the imidazole rings (Fig. 1B). Thus, they can be assigned to the C2 protons of the histidyl imidazoles coordinated to the heme irons. This means that all of the coordinated histidines could be detected as separate signals. They were designated as Im_1–Im_8 from the low to high field. A sharp peak at about 8.9 ppm also disappeared. This can be ascribed to the C2 proton of the non-coordinated histidine (His-67), which agrees with the earlier assignment [6].

The chemical shifts of the C2 signals of the coordinated histidines are plotted as a function of inverse absolute temperature in Fig. 2. Most of them followed Curie's law. In the case of Im_1 and Im_3, however, the tendency was different. Since the microscopic redox potentials of cytochrome c_3 are pH dependent [7], and

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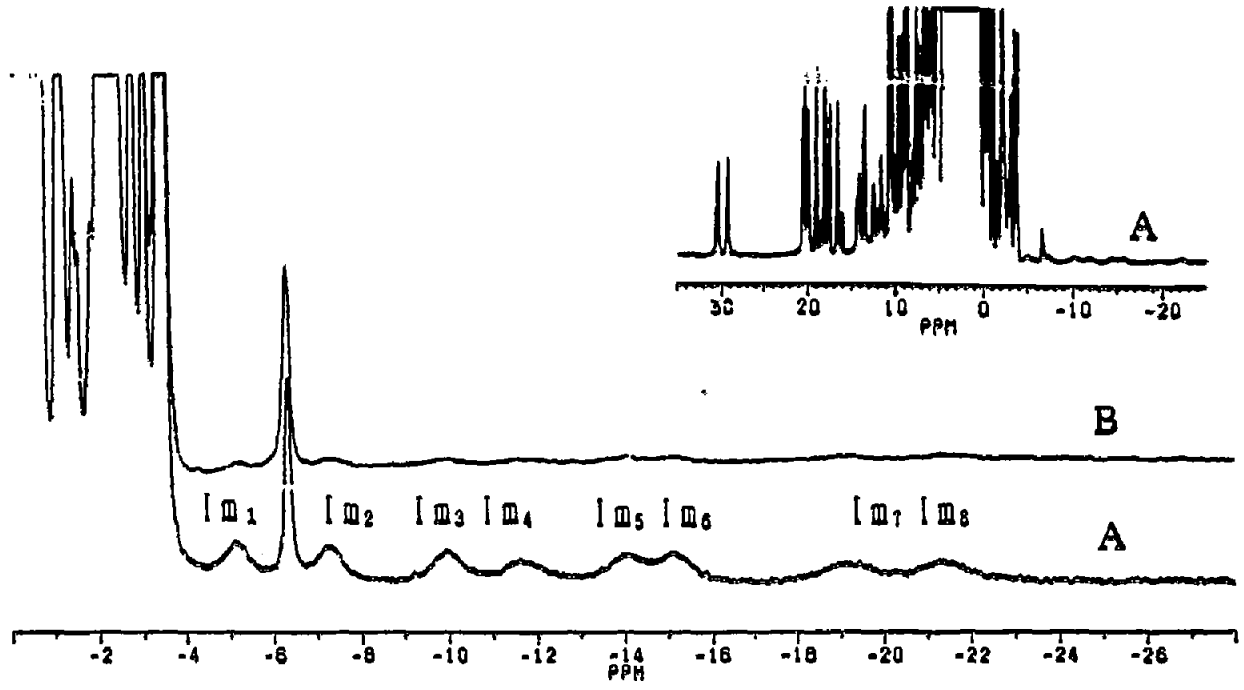

Fig. 1. 400 MHz ^{1}H NMR spectra of *Dv*MF cytochrome c_3 at p^{2}H 7.0 and 30°C. (A) Non-deuterated. (B) Specifically deuterated at the C2 position of histidyl imidazole. (Insert A) Whole spectrum of the non-deuterated cytochrome c_3.

deprotonation of the coordinate imidazole in the pH range 8–9 was proposed for cytochrome *c'* [8], the chemical shifts of C2 signals were examined as a function of p^{2}H, as shown in Fig. 3. The change was small in the pH range from 4.8 to 10. However, the titration curves are similar to each other for the pairs, Im_3 and Im_7, and Im_4 and Im_8, and for Im_1, Im_2 and Im_6.

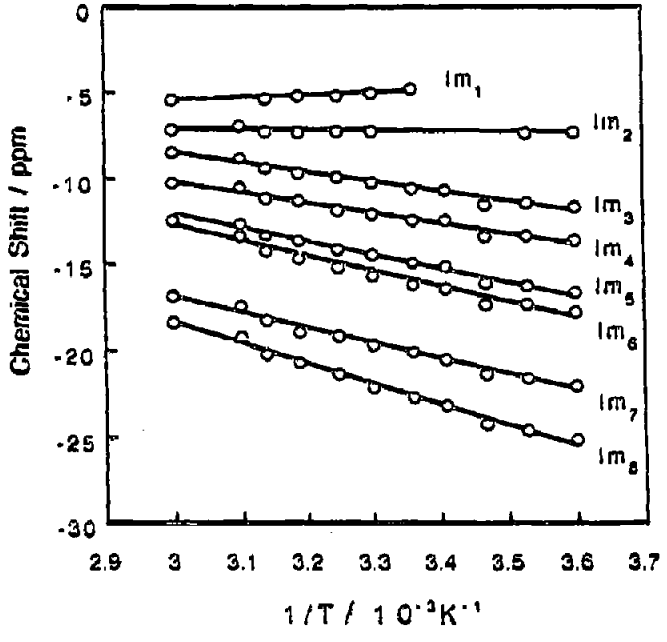

Fig. 2. Temperature dependence of the chemical shifts of the C2 protons of the coordinated histidyl imidazoles at p^{2}H 7.0. For the labels, see Fig. 1.

4. DISCUSSION

All of the C2 protons of the coordinated imidazole rings of *Dv*MF cytochrome c_3 were identified unequivocally in this work. Their chemical shifts were unusually high. It was shown in model experiments that the chemical shift of the C2 proton of imidazole ring shifts upfield by 5–19 ppm on deprotonation (imidazolate formation) [9,10]. In the case of bis5-methylimidazole protoporphyrin IX and monocyano-mono5-methylimidazole protoporphyrin IX, it changed from −10.44 to −18.20 ppm [9] and 2.3–16.3 ppm [10] at 25°C, respectively. Furthermore, the analysis of the orientation of the magnetic susceptibility tensor in cyanometmyoglobin showed that the tilt of the ligand is very sensitive to the chemical shift of the C2 and C4 protons of the imidazole group [11]. In the crystal structure of *Dv*MF cytochrome c_3, the vectors N$^\epsilon$-Fe of the axial ligands are almost normal to the heme plane, namely, seven of them are at the angle of 85–88° and one is at 83° [3]. Therefore, although the orientation of the magnetic susceptibility tensor may contribute to the unusual shifts to some extent, it cannot be the major factor for the scattered unusual shifts. A broad signal was found at −29.9, −20.6 and −9.0 ppm for the cyanocomplexes of horseradish peroxidase, cytochrome *c* peroxidase [10] and lignin peroxidase [12], respectively, and was ascribed to the C2 proton of the proximal histidine on the basis of its extremely broad line width. The unfield shift of the signal was interpreted in terms of the imidazolate-like nature of the ligand, which was used to justify the

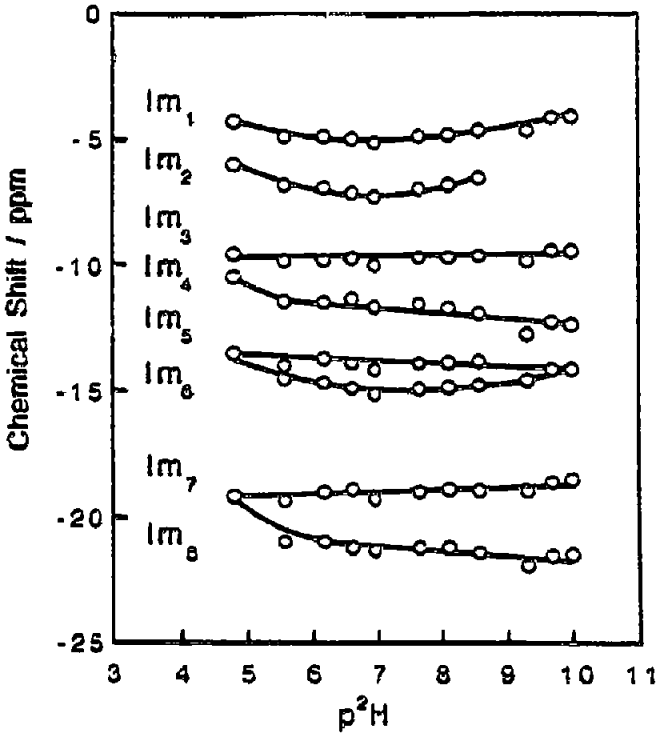

Fig. 3. Titration of the chemical shifts of the C2 protons of the coordinated histidyl imidazoles at 30°C. For the labels, see Fig. 1.

low redox potentials of these proteins [10,12]. An empirical relationship between the chemical shift and the redox potential was also proposed [12]. Since these proteins are in the high spin state under physiological conditions and cytochrome c_3 is in the low spin state, the proposed relationship cannot be directly applied to cytochrome c_3. Nevertheless, it can be concluded from the chemical shifts of the C2 protons that the chemical properties of the imidazole rings coordinated to the four hemes of cytochrome c_3 are quite different from one another, and some of them are expected to carry imidazolate-like nature. The former clearly shows the non-equivalence of the four hemes in cytochrome c_3. The imidazolate-like nature of the ligands might be responsible for the extremely low redox potentials of cytochrome c_3 as a c- type cytochrome. Inspection of the crystal structure gave no clue so far for the interpretation of the abnormal chemical shifts of the ligands.

The temperature dependence of the chemical shifts suggests that the chemical shift change of at least two ligands include structural contributions besides the magnetic one from the irons. This is another piece of evidience for the non-equivalent natures of the coordinated imidazole rings. The p^2H dependence showed that there is neither deprotonation nor protonation of the coordinated imidazole groups in the region from p^2H 4.8 to 10.0. Therefore, any change in the redox potential in this pH range cannot be attributed to the coordinated imidazole groups.

Acknowledgements: We are grateful to Prof. I. Morishima, Kyoto University, for the discussions. This work was partly supported by Grant-in-Aids for General Research (No. 01430024) and for Scientific Research of Priority Area of 'Bioinorganic Chemistry' from the Ministry of Education, Science and Culture.

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