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SPECTRAL AND KINETIC ABNORMALITY DURING THE REDUCTION OF CYTOCHROME c_3 CATALYZED BY HYDROGENASE WITH HYDROGEN

TATSUHIKO YAGI

Department of Chemistry, Shizuoka University, 836, Oya, Shizuoka 422 (Japan)

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(1) Reduction process of cytochrome c_3 by hydrogenase (EC 1.12.2.1) under H_2 was analyzed by means of spectrophotometry. (2) When cytochrome c_3 is in equilibrium with H_2 under reduced pressure, spectral abnormality in the Soret region appeared most significantly at $\frac{1}{4}$ reduction state, less significantly at $\frac{1}{2}$ reduction state, and disappeared at $\frac{3}{4}$ reduction state. (3) The spectral changes during the enzymic reduction of cytochrome c_3 in H_2-saturated solution traced at several wavelengths also showed spectral abnormality in the Soret region at the early stage of reaction. (4) The first-order rate constants for the successive reduction steps from all-ferric to all-ferrous form of cytochrome c_3 was estimated to be $k_1 = 0.061\ s^{-1}$, $k_2 = 0.063\ s^{-1}$, $k_3 = 0.039\ s^{-1}$ and $k_4 = 0.014\ s^{-1}$ (cytochrome c_3: 2 μM; hydrogenase: 2 nM, and at 20°C, pH 7.0). (5) Strong interaction is suggested between hemes 3 and 4 (for the refined structure and heme-numbering, see Higuchi, Y., Kusunoki, M., Matsuura, Y., Yasuoka, N. and Kakudo, M. (1984) J. Mol. Biol. 172, 109–139). (6) The first electron from hydrogenase is supposed to be transferred to these hemes and delocalized between them, and the second electron, among hemes 3, 4 and 1. (7) The characteristic behavior in the enzymic reduction of cytochrome c_3 is different from that in non-enzymic reduction.

Introduction

Cytochrome c_3 is a tetrahemoprotein electron carrier present in sulfate-reducing bacteria, *Desulfovibrio*. It has a very negative redox potential, and is a natural electron carrier for the enzyme hydrogenase [1,2]. Each heme in cytochrome c_3 molecule is liganded by two imidazoles of histidyl residues [3,4]. These structural features are different from most *c*-type cytochromes found either in eucaryotic mitochondria or in procaryotes [5]. Intramolecular heme-heme interaction is observed by physicochemical techniques such as ESR [6], NMR [7,8] and Mössbauer spectrometry [9].

Tabushi et al. [10] observed spectral abnormality during non-enzymic reduction of cytochrome c_3 with aqueous sodium dithionite solution and estimated the rate constants for the successive reduction of cytochrome c_3 from the all-ferric state to the all-ferrous state. They concluded that the spectral abnormality is due to formation of an intermediate having its own characteristic spectrum. Kinetics of non-enzymic reduction of cytochrome c_3 have also been studied by Favaudon et al. [11] and Van Leeuwen et al. [12].

This paper discusses spectral abnormality and reduction kinetics during the enzymic reduction of cytochrome c_3 by hydrogenase under the atmosphere of H_2. Different features were observed between the enzymic and non-enzymic reduction of cytochrome c_3.

Materials and Methods

Cytochrome c_3 and hydrogenase. Cells of *Desulfovibrio vulgaris* Miyazaki F (Registered num-

ber, IAM 12604, the Institute for Applied Microbiology, University of Tokyo) were grown and harvested, and then cytochrome c_3 and hydrogenase (hydrogen : ferricytochrome c_3 oxidoreductase, EC1.12.2.1) were purified as described previously [1,13].

Enzymic equilibrium of cytochrome c_3 with hydrogen. A glass reactor used in these experiments is made of Pyrex glass having a quartz-made optical cell (light path, 10 mm) as a side arm. The volume of the reactor is 103.86 ml. The reactor also has two stopcocks connected with a capillary tube whose volume is 0.178 ml. A 5.0 ml portion of a solution containing 0.11 μM hydrogenase, 2 μM cytochrome c_3 and 0.02 M phosphate buffer (pH 7.0) was placed in the maincompartment of the reactor, and the gas phase was evacuated to remove air by a vacuum line system equipped with a Hickman diffusion pump. Then the spectrum of ferricytochrome c_3 was recorded. H_2 gas was then introduced into the capillary part of the reactor, the reactor was detached from the vacuum line, and the H_2 in the capillary was made to diffuse to the main part of the reactor, so that the partial pressure of H_2 was reduced to 0.00179 of that which had been originally introduced in the capillary. This technique, therefore, enables us to introduce H_2 precisely at pressure as low as 2 Pa ($2 \cdot 10^{-5}$ atm). On introducing H_2, the enzymic reduction of cytochrome c_3 began. It took about an hour to attain redox equilibrium between cytochrome c_3 and H_2. This procedure was repeated several times, and finally cytochrome c_3 was fully reduced by direct introduction of H_2 to the reactor at a pressure of over 40 kPa (300 Torr).

Deoxygenation of gases. Gas was deoxygenated by passing through a Gasclean column (Type, GC-RP; 25 × 450 mm; Nikka Seiko Co., Tokyo) which reduces O_2 concentration to 0.01 ppm.

Deoxygenation of hydrogenase solution for use in kinetic studies. A solution of hydrogenase (20 nM) containing 2 μM cytochrome c_3 was made anaerobic by bubbling deoxygenated N_2 for more than 12 h, and kept under the stream of deoxygenated N_2 during the experiments. The cytochrome added has effect to stabilize hydrogenase during the experiments. Insufficient deoxygenation (i.e., bubbling for less than 1 h) did not give reproducible results.

Monitoring of enzymic reduction of cytochrome c_3. A 3.0 ml portion of cytochrome c_3 solution in 0.02 M phosphate buffer (pH 7.0) was placed in an optical cell (light path, 10 mm) kept in a spectrophotometer, and made to be saturated with H_2 by bubbling deoxygenated H_2 for 15 min. To start the reaction, 0.3 ml deoxygenated hydrogenase solution was sucked in a syringe, and ejected into the cell using a specially designed needle [14] to cause immediate mixing of the content in the cell. The inlet of the bubbling H_2 was removed immediately from the cell content, over which the flow of the deoxygenated H_2 was made to sweep during the experiment. Enzymic reduction of cytochrome c_3 starts immediately after mixing, and can be monitored at various wavelengths by a recorder. The reduction percentage of cytochrome c_3 was estimated from the absorbance at 552 nm where no spectral abnormality had been observed as will be shown later in Results.

Estimation of kinetic parameters. In the course of the enzymic reduction of cytochrome c_3, there are three reduction intermediates, III_3II, III_2II_2 and $III\,II_3$, where III and II stand for the ferri and ferro forms of the hemes in cytochrome c_3. Here, we did not distinguish individual hemes in the molecule. The enzymic reduction of cytochrome c_3 in the H_2-saturated solution can, therefore, be expressed by a series of reaction steps as mentioned in Eqn. 1. Each elementary reaction can be assumed to be of first order with respect to cytochrome c_3 when the concentration of the cytochrome is lower than K_m. k_n is the pseudo-first-order rate constant (at a fixed concentration of hydrogenase) of the nth step:

$$III_4 \xrightarrow{k_1} III_3II \xrightarrow{k_2} III_2II_2 \xrightarrow{k_3} III\,II_3 \xrightarrow{k_4} II_4 \qquad (1)$$

From the differential equations set up for the individual steps, one can calculate the reduction percentage, i.e., the percentage of total ferrohemes in the cytochrome at time t, as Eqn. 2 [10]:

$$\text{Reduction percentage} \times 0.01 =$$

$$1 + \{k_1^3 - k_1^2(2k_2 + k_3 + k_4) + k_1(k_3k_4 + 2k_2k_4 + 3k_2k_3)$$

$$-4k_2k_3k_4\}\{4(k_2 - k_1)(k_3 - k_1)(k_4 - k_1)\}^{-1} e^{-k_1t}$$

$$+\frac{k_1(3k_3k_4-2k_2k_3-k_2k_4+k_2^2)}{4(k_2-k_1)(k_3-k_2)(k_4-k_2)}e^{-k_2t}$$

$$+\frac{k_1k_2(k_3-2k_4)}{4(k_3-k_1)(k_3-k_2)(k_4-k_3)}e^{-k_3t}$$

$$+\frac{k_1k_2k_3}{4(k_4-k_1)(k_4-k_2)(k_4-k_3)}e^{-k_4t} \qquad (2)$$

A set of the four constants which precisely reproduced the time-course curve of the enzymic reduction was obtained by the procedure given by Tabushi et al. [10].

Results

Absorption spectra. The absorption spectra of partially and fully reduced cytochrome c_3 at 25°C are shown in Fig. 1. In the spectra in the $\alpha \sim \beta$ peak region (Fig. 1), four isosbestic points are observed at 561, 542, 532 and 507 nm, suggesting that absorbance is additive in this region. Spectrum 1 was considered to be of 11% reduced cytochrome c_3, because it is very similar to the curve composed of 11% curve R and 89% curve O (Fig. 2), where curves R and O stand for the spectra of the ferro and ferri forms of cytochrome c_3, respectively. Similarly, spectra 2, 3 and 4 were considered to be of 26%, 50% and 80% reduced cytochrome c_3, respectively (Fig. 2).

On the other hand, there was no isosbestic point in the Soret region (Fig. 1). Spectrum 1 is significantly deviated from the curve composed of 11% curve R and 89% curve O, so was spectrum 2 deviated from the curve composed of 26% curve R and 74% curve O (Fig. 2). The deviation of spectrum 3 from the composed spectrum for 50% reduction became less significant, and spectrum 4 was almost identical with that of the composed spectrum for 80% reduction (Fig. 2).

Upon equilibrium, reduction percentages at 3.22, 8.87 and 64.2 Pa H_2 were 26%, 50% and 80%, respectively. The standard redox potential of cytochrome c_3 calculated from these data was −294 mV, which agreed well with the value as reported previously [1,2].

Michaelis constant of ferricytochrome c_3 for hydrogenase. From the initial rates of the enzymic reduction of cytochrome c_3 at different concentrations monitored at 552 nm at 20°C, K_m of ferricytochrome c_3 was estimated to be 4.0 μM.

Enzymic reduction of cytochrome c_3 under H_2. Fig. 3 illustrates time-course curves of the absorbance changes at different wavelengths upon rapid mixing of deoxygenated enzyme solution and H_2-saturated cytochrome c_3 solution in an optical cell at 20°C. Here the reduction percentages at 11.3, 21.8, 44.3 and 98.0 s from the start of the reaction were 11, 26, 50 and 80%, respectively. The absorbances at different wavelengths at their corresponding time were overlapped the spectra of the corresponding reduction percentages (Fig. 2). As shown in this figure, the spectra of the intermediate states during the en-

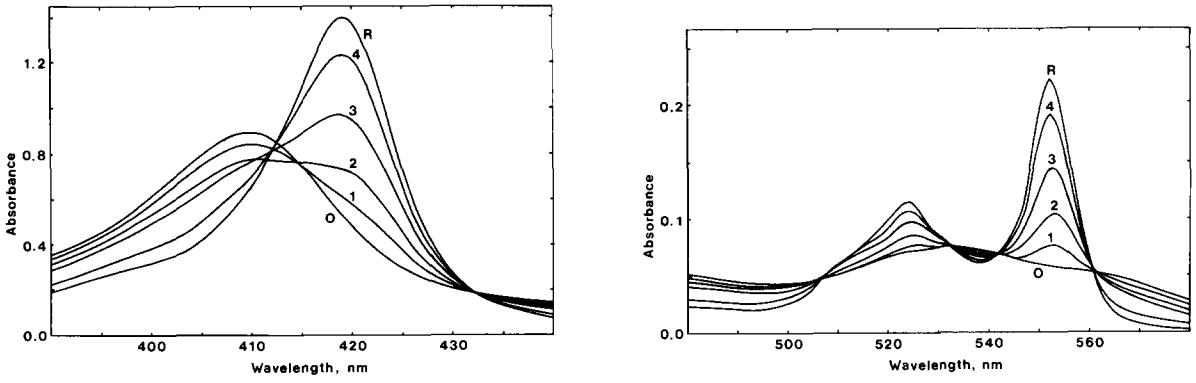

Fig. 1. Absorption spectra of cytochrome c_3 in various redox states. Detailed procedure to record the spectra is given in the text. Curve O: spectrum of ferricytochrome c_3 recorded before the introduction of H_2 to the reactor; curve R: spectrum of ferrocytochrome c_3 recorded after full reduction under 40 kPa H_2; curve 1: spectrum of cytochrome c_3 recorded under 2.92 Pa H_2 and before attaining the equilibrium; curves 2, 3 and 4: spectra of cytochrome c_3 in equilibrium under 3.22, 8.87 and 64.2 Pa H_2, respectively.

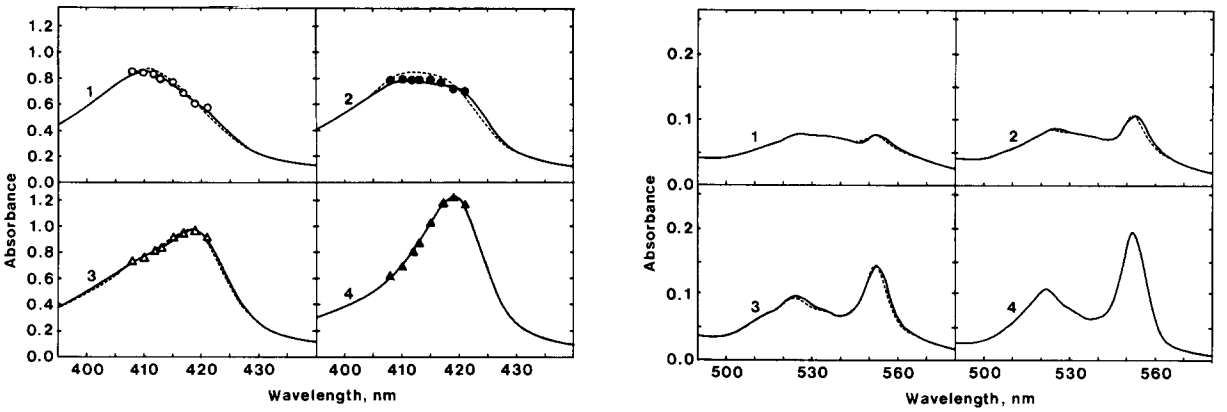

Fig. 2. Absorption spectra of cytochrome c_3 in intermediary redox states. Solid lines: observed spectra, reproduced from Fig. 1. Curves 1–4 represent spectra of cytochrome c_3 in 11, 26, 50 and 80% reduced states, respectively. Dotted lines: composed spectra from curves R and O in Fig. 1. The plots denote absorbances during the enzymic reduction of cytochrome c_3 at designated time, ○, 11.3 s, ●, 21.8 s, △, 44.3 s and ▲, 98.0 s, from the start of the reaction at 20°C.

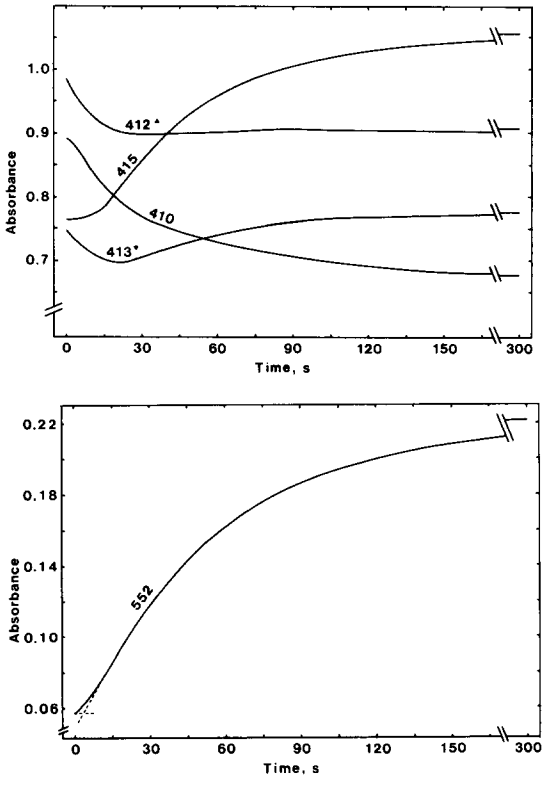

Fig. 3. Absorbance change during the enzymic reduction of cytochrome c_3. A 0.3 ml deoxygenated hydrogenase solution (20 nM) was ejected into the cell containing 3.0 ml of H_2-saturated cytochrome c_3 solution (2 μM) at 20°C as mentioned in the text. The absorbances at 412 and 413 nm were shifted upward and downward by 0.1 absorbance unit, respectively. The absorbance changes at 408, 417, 419 and 421 nm are not reproduced here.

zymic reduction of H_2-saturated cytochrome c_3 solution coincide well with those of the partially reduced states which are in equilibrium with H_2. These results indicate that under the H_2-saturated conditions the hydrogenase-catalyzed reduction of cytochrome c_3 traces exactly the same inter-

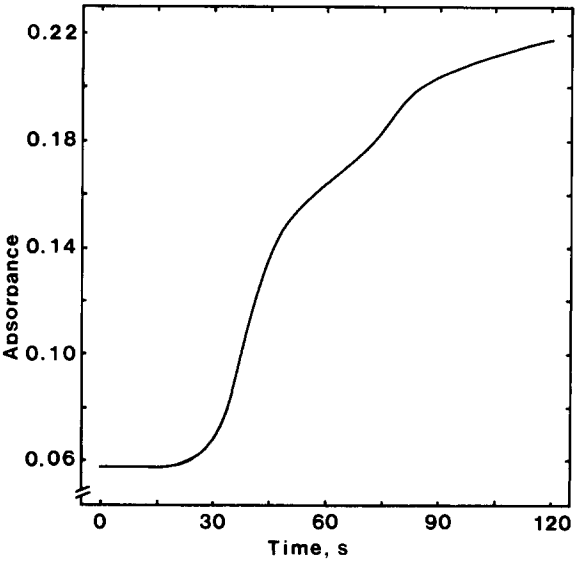

Fig. 4. Absorbance change during the enzymic reduction of cytochrome c_3 with insufficiently deoxygenated hydrogenase. The reaction conditions were the same as those in Fig. 3, except that the concentration of hydrogenase was 10-times as concentrated, and the hydrogenase solution had been deoxygenated for only 30 min before the experiments.

mediary redox species which are in equilibrium with H_2 at low pressure.

The time-course curves of the enzymic reduction of cytochrome c_3 were reproducible as far as the enzyme solution had been deoxygenated completely before the experiments, but irregular time-course curves were observed when deoxygenation had been insufficient as shown in Fig. 4.

Kinetics of enzymic reduction of cytochrome c_3 under H_2. From the time-course curve of the enzymic reduction of cytochrome c_3 monitored at 552 nm (Fig. 3), four kinetic constants for the successive four steps (Eqn. 1) were obtained as follows:

$k_1 = 0.061\ s^{-1}$, $k_2 = 0.063\ s^{-1}$,

$k_3 = 0.039\ s^{-1}$, $k_4 = 0.014\ s^{-1}$

(at a hydrogenase concentration of 2 nM, and at pH 7.0, 20°C).

Discussion

It has been observed by physicochemical techniques [6–9] that intramolecular heme-heme interaction exists in cytochrome c_3. Tabushi et al. [10] observed that during non-enzymic reduction of cytochrome c_3 with aqueous dithionite solution, changes in absorbances at wavelengths covering Soret region behaved abnormally. For example, A_{413} (the absorbance at 413 nm) during the reduction is lower than the sum of A_{413} of the ferro-form fraction and A_{413} of the ferri-form fraction, but A_{419} during the same process is higher than the sum of A_{419} of the ferro-form fraction and A_{419} of the ferri-form fraction. The abnormality in absorbances can be accounted for assuming that there is an intermediate state which has characteristic absorption spectrum. Concentration of the intermediate was maximum at the state between $\frac{1}{4}$ and $\frac{3}{4}$ reduction. They observed no spectral abnormality at wavelengths in the other regions.

In the present study, we measured absorption spectra of partially reduced species of cytochrome c_3 which are in equilibrium with H_2 in the presence of hydrogenase. As in the case of the non-enzymic reduction of cytochrome c_3, the spectral abnormality was observed mainly in the Soret region. The observed spectrum at $\frac{1}{4}$ reduction was significantly deviated from the spectrum composed of $\frac{1}{4}$ curve R and $\frac{3}{4}$ curve O (Fig. 2). The deviation of the observed spectrum from the composed one was less significant at $\frac{1}{2}$ reduction state, and negligible at reduction states over $\frac{3}{4}$; i.e., the spectral abnormality appears when one or two of the four hemes are in the ferro form, and disappears when the third heme is reduced. Since the Soret band reflects the excitation of delocalized π-electrons to unoccupied levels of the porphyrin rings of similar angular momentum [15], the spectral abnormality in this region can be accounted for by the assumption that there are two hemes which interact strongly, that the interaction involving the third heme is less significant, and that the interaction involving the fourth heme is negligible; here the first, the second, etc., refer to the reduction order of the hemes in the molecule.

From the estimated rate constants during the enzymic reduction of cytochrome c_3, the percentages of the individual redox species, III_4, III_3II, III_2II_2, $III\,II_3$ and II_4 were calculated according to the rate equations [10]. The concentration of III_3II was found to become maximum (36% of the total redox species) after 20 s from the start of the reaction. It is noteworthy that A_{413} was minimum at the same moment (Fig. 3). This also supports the assumption that the spectral abnormality in the Soret region is mainly due to the formation of III_3II.

Studies on the reduction kinetics of cytochrome c_3 have so far been conducted by non-enzymic techniques. Favaudon et al. [11], using cytochrome c_3 from *D. vulgaris* Hildenborough, observed biphasic reduction kinetics, i.e., the initial rapid phase up to 50% reduction was followed by the slow phase; the ratio of the rate constants of respective phases being 3.2 : 1. Van Leeuwen et al. [12] measured the rate constant during non-enzymic reduction of cytochrome c_3 with methylviologen radical by pulse radiolysis technique. Tabushi et al. [10], using cytochrome c_3 from *D. vulgaris* Miyazaki F, analyzed the successive reduction steps from all-ferric to all-ferrous state, and estimated the ratio of the four rate constants, $k_1 : k_2 : k_3 : k_4$, to be 5 : 3 : 2.2 : 0.4.

The kinetic characteristics for the enzymic reduction of cytochrome c_3 with H_2 differ somewhat

from those in the non-enzymic reduction. During the successive enzymic reduction from all-ferric to all-ferrous state, k_2 is larger than k_1. If four hemes are independent of each other, the ratio of the four constants should be 4:3:2:1, but the observed ratio was 2.9:3:1.9:0.7. This means that when the first heme is reduced, the second heme becomes more easily reducible, i.e., the enzymic reduction of the first and the second hemes is a kind of concerted reaction of positive cooperativity. This is also consistent with our assumption that the two strongly-interacting hemes accept the first and second electrons from H_2. Difference in kinetic features between the enzymic and non-enzymic reduction may be explained if the heme which accepts the first electron in the enzymic reduction is not accessible from the molecular surface in the non-enzymic collision mechanism.

If the four hemes are equivalent and non-interacting each other, difference of the apparent midpoint potentials must be 25.0 mV between $E_1^{0\prime}$ and $E_2^{0\prime}$, 20.7 mV between $E_2^{0\prime}$ and $E_3^{0\prime}$, and 25.0 mV between $E_3^{0\prime}$ and $E_4^{0\prime}$ [16]. The concerted reduction of the first and the second hemes means that the reduction of the first heme makes the second more easily reducible, i.e., the difference between $E_1^{0\prime}$ and $E_2^{0\prime}$ must be less than 25.0 mV. The independent reduction of the second and the third hemes suggests that the difference between $E_2^{0\prime}$ and $E_3^{0\prime}$ is close to 20.7 mV. This conclusion is not in accordance with that obtained from electrochemical measurements [16], probably due to heterogeneous kinetics in the latter.

According to the three dimensional structure of cytochrome c_3 from *D. vulgaris* [4,17], strong π-π interaction between hemes 3 and 4* is expected through phenylalanine 20, whose aromatic ring is inserted between the plane of heme 3 and an imidazole ligand of heme 4 (Fig. 5). This interaction seems to be very strong, because even with insufficiently deoxygenated hydrogenase, the reduction up to 50% proceeded by one step, followed by delayed reduction of the remaining hemes as shown in Fig. 4. Moreover, the distance between the plane of heme 3 and the imidazole ring is only about 0.6 nm, and phenylalanine 20 is one

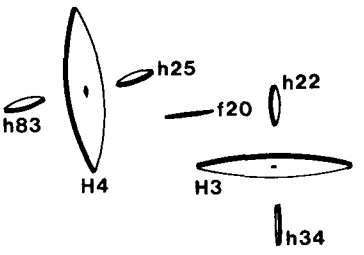

Fig. 5. Schematic representation of the orientation of hemes 3 and 4. H, h and f stand for heme, histidine and phenylalanine, respectively.

of the evolutionarily conserved amino acid residues among all known cytochrome c_3 molecules including cytochrome *c*-551.5 (a trihemoprotein) from *Desulfuromonas acetoxydans*. Moderate interaction between hemes 4 and 1 is expected because they are in close proximity. On the other hand, interaction between hemes 3 and 2, which are also in proximity, seems not to be very significant, because their orientation is not suitable for π-π interaction. Heme 2 seems not to have an important role during evolution, because it is not present in cytochrome *c*-551.5, whose amino acid sequence [18] is homologous to that of cytochrome c_3 except that the former does not have a heme-binding sequence for heme 2 of the latter. Interaction among other hemes may be much weaker because they are more distant from each other. Based on these structural characteristics and the spectral and kinetic abnormality during the enzymic reduction, one can suppose the mechanism of the enzymic reduction of cytochrome c_3 as follows. When the first electron is transferred from hydrogenase to cytochrome c_3, it must be delocalized between Hemes 3 and 4 by π-π interaction through phenylalanine 20 to cause spectral abnormality. The first electron will increase the electronic conduction among hemes in the molecule to make k_2 larger than k_1. It has been experimentarily confirmed that ferrocytochrome c_3 is more electrically conductive than ferricytochrome c_3 [2]. When the second electron is accepted, reduction of the heme-3-heme-4 pair completes, but electron delocalization among hemes 3, 4 and 1 causes some spectral abnormality of the half reduced cytochrome c_3. When the third electron is accepted to reduce fully hemes 3, 4 and 1, there

* Heme numbering is defined in Ref. 4.

could be no significant interaction with the remaining one, heme 2, so that no spectral abnormality could be observed. Delocalization of electrons in the cytochrome c_3 molecule must be much faster than enzymic reduction rate, since the enzymic reduction exactly traces the same intermediary species which is in equilibrium with H_2 of low pressure (Fig. 2). Xavier and his collaborators also concluded rapid intramolecular electron exchange between at least two hemes from their NMR redox studies [8].

In conclusion, heme-heme interaction was suggested during the enzymic reduction of cytochrome c_3 by hydrogenase, but the reduction process is different from that observed during the non-enzymic reduction with aqueous sodium dithionite solution. These observations will provide a clue for better understanding of the mechanism of the reaction of cytochrome c_3 and hydrogenase.

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