Electrochemical Behavior of Cytochrome c_3 of *Desulfovibrio vulgaris*, Strain Miyazaki, on the Mercury Electrode

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Abstract: Potentiometric, pulse polarographic, and cyclic voltammetric techniques were used to investigate the electrochemical behavior of cytochrome c_3 of *Desulfovibrio vulgaris*, strain Miyazaki, on mercury electrode. The electrode reaction of cytochrome c_3, which contains four hemes in the molecule, is reversible and diffusion controlled with a diffusion coefficient of 0.94 $\times 10^{-6}$ cm^2 s^{-1} for the ferri form and 0.71 $\times 10^{-6}$ cm^2 s^{-1} for the ferro form. The formal potential of the ferri- and ferrocytochrome c_3 couple is -0.528 V (-0.287 V vs. NHE) and the polarographic half-wave potential is -0.527 V. The electrode reaction rate constant of cytochrome c_3 is too fast to be measured by the present electrochemical methods. It is estimated to be 0.1 cm s^{-1} or greater. A large Nernst slope and broad voltammograms with smaller peak current than expected from a four-electron process suggested that the electrode reactions of cytochrome c_3 are four one-electron processes and the hemes in the molecule are nonequivalent and noninteracting. The spacings between the standard potentials of the adjacent hemes are about -50, -20, and -40 mV, respectively.

Cytochrome c_3 is a multiheme protein isolated from sulfate-reducing bacteria, *Desulfovibrio*.[2-10] It is similar to mammalian cytochrome *c* in molecular weight and in absorption spectrum but has a very negative redox potential among cytochromes. The number of hemes in cytochrome c_3 was determined to be four per molecule by chemical analysis,[9,11] ESR spectroscopy,[12,13] and NMR spectroscopy.[14,15] The amino acid sequence of cytochrome c_3 also indicates four possible heme binding sites.[16,17]

In the course of the reoxidation of cytochrome c_3 from *D. vulgaris* with oxygen after chemical reduction, a stable intermediate at half-reoxidized state was observed in the ESR measurements.[12,18] This intermediate, distinguishable from the ferri low-spin form by a distinct narrowing at g_z and g_x but, otherwise, with the same *g* values, was attributed to a decrease in the interaction between the hemes. At 8.5 K the four different hemes were detected and four unique sets of *g* values indicated that the hemes in the protein molecule are nonequivalent.[13] Similar results were also noted on *D. vulgaris*, strain Miyazaki, by Mössbauer spectroscopy.[19] The redox cycling by chemical reduction and reoxidation with oxygen revealed that the individual hemes were reoxidized at different rates indicating dissimilar hemes in the measurements at 8.5 K.[13]

McDonald and his co-workers[14] have observed in the NMR measurements of cytochrome c_3 from *D. vulgaris* that resonances of three different oxidation states appear to develop in turn as oxidation proceeded. Their tentative conclusion is that the oxidation state I represents oxidation of one heme, the oxidation state II represents oxidation of two hemes, and the oxidation state III corresponds to the fully oxidized protein. When the oxidized form is more than one-third of cytochrome c_3, all three oxidation states coexist. That is, electron exchange between the reduced state and the three oxidized states is too slow to cause the exchange averaging of the respective NMR spectra despite the fact that the hemes are close together. Dobson et al.[15] have observed results similar to those of McDonald et al. in NMR studies. The reduction titration of cytochrome c_3 with dithionite by monitoring the NMR spectra showed that there must be four hemes per molecule which are reduced in two-electron steps separated in midpotential by about 50 mV at 25 °C.[15]

The physicochemical properties of cytochrome c_3 extracted from *D. vulgaris*, strain Miyazaki, have been examined extensively in our laboratory.[9] The amino acid composition is only slightly different from that of cytochrome c_3 extracted from *D. vulgaris*, strain Hildenborough, and both heme proteins have the lowest redox potentials ever reported among the *c*-type cytochromes. Cytochrome c_3 is a natural electron acceptor for *Desulfovibrio* hydrogenase (hydrogen: ferricytochrome c_3 oxidoreductase, EC 1.12.2.1.), and is reduced reversibly by molecular hydrogen in the presence of hydrogenase.[20] It has been demonstrated by Yagi et al.[21] that cytochrome c_3 is electrochemically active and interacts directly with a glassy carbon electrode as an electron carrier. In our previous work,[22] we found that the electron-transfer reaction of cytochrome c_3 is reversible at the mercury electrode surface, which is the first instance of observing a reversible electrode reaction for a heme protein. Yeh and Kuwana[23] also reported a reversible electrode behavior of cytochrome *c* on a tin-doped indium oxide electrode.

In the present work, we have further examined the electrode behavior of cytochrome c_3 of *D. vulgaris*, strain Miyazaki, at mercury electrode by pulse and differential pulse polarography, cyclic voltammetry, and potentiometry.

Experimental Section

Materials. The procedures for cultivation of *D. vulgaris*, strain Miyazaki, and the purification of cytochrome c_3 and hydrogenase (hydrogen: ferricytochrome c_3 oxidoreductase, EC 1.12.2.1.) from bacterial cells were given elsewhere.[9,24] All preparations were stored in a refrigerator at -20 °C until used.

The purity of cytochrome c_3 was expressed in terms of the ratio of light absorbance of the α peak at 552 nm for the ferro form to that at 280 nm for the ferri form. This ratio (purity index) is estimated to be 2.98 for pure cytochrome c_3 from our measurements. The purity index of cytochrome c_3 for the present experiments was in the range 2.4–2.9;[7] that is, the purity of cytochrome c_3 used in the present experiments was over 80%. There was no observable difference in the results of electrochemical measurements with this cytochrome c_3. In the calculation of cytochrome c_3 concentration a molar absorptivity of 112 000 (28 000 for each heme) at 552 nm was used after the complete reduction of ferricytochrome c_3 to the ferro form by sodium dithionite.[9] Absorption spectra were obtained with a Cary Model 17 or a Hitachi Model 124 spectrophotometer. Anaerobic measurements were made in quartz spectrophotometric cells of 1.0-cm light path under nitrogen.

Both potassium dihydrogen phosphate and disodium hydrogen phosphate were a pH standard grade obtained from Wako Pure Chemical Co. and were used without further purification. Solutions

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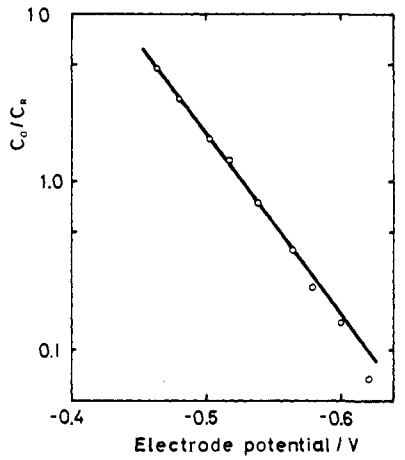

Figure 1. Equilibrium potentials of the ferri- and ferrocytochrome c_3 couple in 0.03 M phosphate buffer solution at pH 7.0.

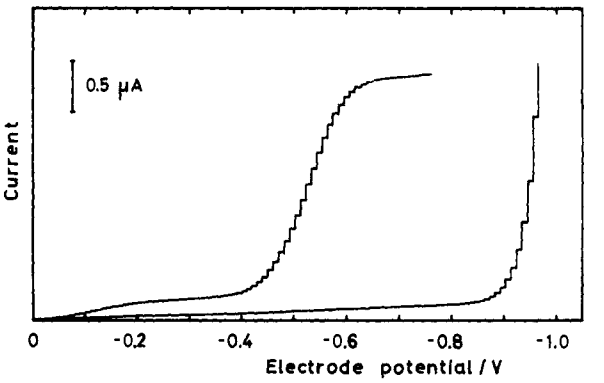

Figure 2. Normal pulse polarograms of the reduction of ferricytochrome c_3 and of the base solution (0.03 M phosphate buffer): 1.24×10^{-4} M cytochrome c_3; m = 2.82 mg/drop; sampling time 50 ms.

were prepared with doubly distilled water. The stock solution was 0.3 M phosphate buffer at pH 7.0 and was added to the preparation of cytochrome c_3 prior to the measurements.

Polarographic and cyclic voltammetric studies were carried out with a Fuso Polarograph Model 312 (Fuso and Co., Kawasaki, Japan). A normal pulse mode with varied sampling time (30, 50, and 70 ms from the application of the pulse) and a differential pulse mode with a modulation amplitude of 10 mV were used in the polarographic measurements. The drop time of the dropping mercury electrode was 2 s. In the cyclic voltammetry a Metrohm hanging mercury drop electrode was used.

In the potentiometric measurements, a slow DME (15–20 s/drop) was used as an indicator electrode. The redox potential of the ferri-/ferrocytochrome c_3 system was measured against the saturated calomel electrode with a Takeda Riken Model TR-6854 digital voltmeter.

A three-compartment water-jacketed electrochemical cell was used and each compartment was separated by a porous Vycor glass. The cytochrome solution was deaerated by bubbling purified nitrogen through the solution. Nitrogen was purified by bubbling through a concentrated vanadous solution and then through distilled water. The cell was maintained under nitrogen atmosphere during the electrochemical measurements by flashing the purified nitrogen over the solution.

All potentials were referred to saturated calomel electrode and the measurements were carried out at 25 °C.

Procedure. The stock phosphate buffer solution was added to the cytochrome c_3 solution, which was thoroughly dialyzed against distilled water, so that 0.03 M phosphate solution of pH 7.0 was obtained.

The solution for optical measurements was 3×10^{-6} M cytochrome c_3 in 0.02 M phosphate buffer to which 0.4 unit/mL of the purified hydrogenase was added. A Thunberg optical cell was connected to a high-vacuum system and the solution was fully deoxygenated by the freeze–thaw cycles with liquid nitrogen. The fully enzymatically reduced cytochrome c_3 spectra were recorded after the reduction of cytochrome c_3 by hydrogen at 15 Torr. Then the solution was frozen and the pressure of hydrogen was reduced to 1×10^{-3} Torr to obtain the partially oxidized form. Ferrocytochrome c_3 changed to the ferri form by releasing hydrogen. The fully oxidized form was obtained by air oxidation.

In the electrochemical measurements, the cytochrome c_3 solution was transferred to the electrochemical cell of 2-mL volume and deaerated for 15–20 min by bubbling with purified nitrogen. The trace amount of oxygen in the solution which still remained after a prolonged deaeration was completely eliminated by the controlled enzymatic reduction by hydrogen with hydrogenase. Most electrochemical measurements were carried out after a simple deaeration by nitrogen for 15–20 min. In the potentiometric measurements, ferricytochrome c_3 was reduced enzymatically with the controlled amount of hydrogen to the ferro form step by step in the presence of a small amount of hydrogenase and then reoxidized to the ferri form by oxygen. The absorbance caused by the added hydrogenase was not detectable in the spectrophotometric measurements. The electrochemical measurements were not influenced by the added hydrogenase, either. The ratio of the ferri form to the ferro form was determined from the polarographic limiting currents (the ferro form from the anodic limiting current and the ferri form from the cathodic limiting current). The initial potentials of the pulse polarography for the determination of the ratio of ferri to ferro form were the equilibrium potentials of the ferri-/ferrocytochrome c_3 system. In the absence of the ferro form in the solution, the initial potential was −0.300 V (vs. SCE) and that for the scan-reversed pulse polarography was in the limiting current region, e.g., −0.70 to −0.75 V (vs. SCE). In the cyclic voltammetric studies, the scan rate ranged from 20 to 200 mV s^{-1}. The peak currents were measured from the base line obtained by extrapolating the background current. The current readings in the pulse polarographic measurements were the average current between 20 and 40, 40 and 60, and 60 and 80 ms, respectively, from the beginning of the pulse. In the differential pulse measurements, the modulation amplitude was 10 mV.

Results

Potentiometry. In the potentiometric study, the plot of log (c_0/c_R) vs. electrode potential produces a straight line with a Nernst slope of 93 mV as shown in Figure 1, where c_0 and c_R represent the concentration of the heme in ferri form and that in ferro form in the solution, respectively, and these concentrations were determined from the cathodic and anodic limiting currents in the normal pulse polarographic measurements. The apparent formal potential, which is the mean redox potentials of the four individual hemes in the molecule, is found to be $-(0.528 \pm 0.001)$ V.

Normal Pulse Polarography. Cytochrome c_3 produces a single well-defined reduction wave with a small preceding wave at the dropping mercury electrode as shown in Figure 2. The half-wave potential, $E_{1/2}$, is −0.527 V (−0.286 V vs. NHE) and the plot of log $[i/(i_{dc} - i)]$ vs. electrode potential produces a straight line with an inverse slope of 0.085 V from which the apparent number of electrons involved in the electron-transfer step is calculated to be less than unity, where i is the cathodic current for the reduction of ferri form at a given potential and i_{dc} is the cathodic limiting current. The current-potential curves for the solutions containing both ferri and ferro forms were symmetrical about the inflection point, at the potential of $(i_{dc} + i_{da})/2$, which was almost identical with the half-wave potential, where i_{da} is the anodic limiting current for the oxidation of ferro form in the solution. These results suggest that the electrode reaction of cytochrome c_3 is reversible pulse polarographically, even though the apparent number of electrons involved in the electrode process is less than unity. The successive enzymatic reduction of ferricytochrome c_3 to the ferro form by hydrogen was investigated by monitoring both the spectral change in the vicinity of the α peak and the polarographic anodic and cathodic limiting currents. The observation of four isosbestic points (560, 542, 532, and 508 nm)

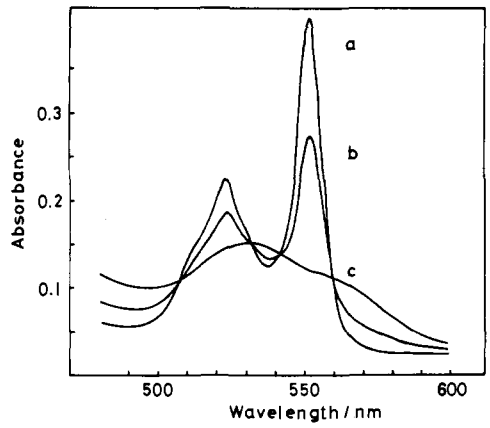

Figure 3. Absorption spectra of cytochrome c_3: (a) ferrocytochrome c_3; (b) partially reduced ferricytochrome c_3; (c) ferricytochrome c_3.

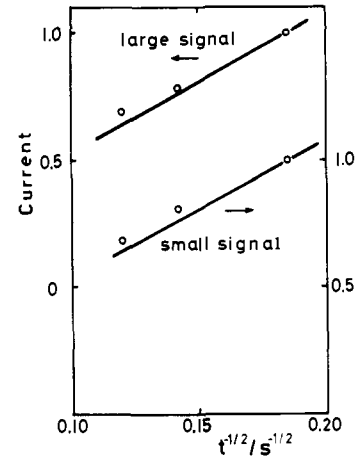

Figure 4. Potentiostatic current-time curves for the reduction of cytochrome c_3 with small and large signals. Concentration of cytochrome c_3, 1.24×10^{-4} M.

indicates that only two types of hemes, ferri and ferro forms, are distingushable in the molecule spectroscopically as shown in Figure 3. The change in the polarographic anodic limiting current was proportional to that in the α peak height. The cathodic limiting current decreased linearly with the increase of the anodic limiting current. The cathodic wave corresponds to the reduction of ferricytochrome c_3 to the ferro form and the limiting current is proportional to its concentration. The anodic wave is for the oxidation of ferrocytochrome c_3 and its concentration is proportional to the anodic limiting current.

The strong adsorption of cytochrome c_3 on the mercury electrode was evidenced not only from the marked depression of the differential capacity curves in the wide potential range but also from the fact that the mercury drop formed in the cytochrome c_3 solution stayed on a mercury pool as a droplet at least for 30 min.

The nature of the preceding wave was investigated by cycling the deoxygenation of the solution with nitrogen and the oxygenation with oxygen. The wave height varied with the amount of oxygen bubbled. After the enzymatic reduction of cytochrome c_3 by hydrogen no wave corresponding to the preceding wave was observable. From these results the preceding wave is very likely to be due to the reduction of oxygen.

The current-time curves at a given potential were measured by using the pulse polarograph with a large potential amplitude (in the potential region of the cathodic limiting current) and with a small potential amplitude (in the potential region at the foot of the polarographic wave) signals. The observed current-time curves were in good agreement with those expected from the diffusion-controlled reversible electrode reaction as shown in Figure 4. The electron-transfer reaction rate between cytochrome c_3 and the dropping mercury electrode is too fast to be determined (a reversible electrode reaction) within the time window of the pulse polarographic measurements (20-80 ms).

The diffusion coefficients of ferri- and ferrocytochrome c_3 calculated from the pulse polarographic data were 0.94×10^{-6} [25] and 0.71×10^{-6} cm^2 s^{-1}, respectively.

Scan-Reversal Pulse Polarography. Oldham and Parry[26] proposed a diagnostic method to distinguish reversible processes from irreversible. The limiting current produced by a pulse polarographic scan, which starts on the diffusion plateau of the reduction process and goes anodically, depends markedly on the reversibility of the electrode reaction. The ratio of the limiting currents for cathodic and anodic scans is expected to be almost unity provided that the electrode reaction is reversible and the diffusion coefficients of both oxidized and reduced forms are equal. Moreover, the half-wave potential on the cathodic scan is equal to that on the reverse scan. In the

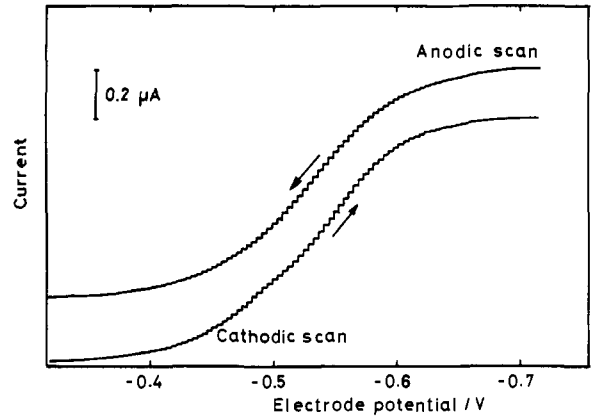

Figure 5. Normal pulse and scan-reversal pulse polarograms of ferricytochrome c_3, 1.24×10^{-4} M. The current axis of the scan-reversal polarogram is shifted upward.

case of an irreversible electrode reaction, on the other hand, the ratio of the limiting currents for cathodic and anodic scan will be about 7 and the half-wave potential on the anodic scan should lie a few millivolts more negative than that on the cathodic scan. The normal pulse and the scan-reversal polarograms of ferricytochrome c_3 are shown in Figure 5, in which the ratio of the limiting currents on the cathodic scan to the anodic scan is almost unity and the half-wave potentials are identical in either direction. These results provide strong evidence for the reversible electrode reaction of cytochrome c_3 on mercury electrode.

Differential Pulse Polarography. The differential pulse polarogram of ferricytochrome c_3 is shown in Figure 6. The polarogram is almost symmetrical with respect to the potential axis at the peak current, −0.522 V. The ratio of the peak current of the differential pulse polarogram to the limiting current of the normal pulse polarogram (i_{dpp}/i_{npp}) is 0.0714, which indicates that the apparent number of electrons involved in the electrode process, n_{app}, is 0.74.[27] The half-peak width is 118 mV from which n_{app} is also calculated to be 0.76,[27] while the values of n_{app} calculated from the potentiometric slope and the logarithmic plot of the pulse polarogram were 0.64 and 0.70, respectively. All of these n_{app} values are in good agreement. The difference between the peak potential of the differential pulse polarogram (−0.522 V) and the half-wave potential of the normal pulse polarogram (−0.527 V) is 0.005 V, which is expected from the theory where the electrode reaction is reversible and the modulation amplitude of the differential pulse polarography is 10 mV.[27]

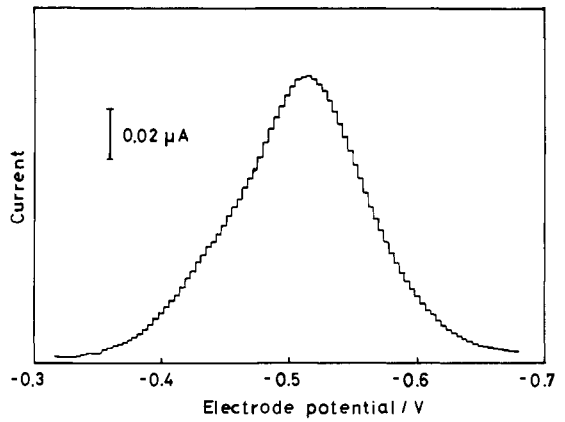

Figure 6. Differential pulse polarogram for the reduction of ferricytochrome c_3: 1.24×10^{-4} M cytochrome c_3; m = 1.99 mg/drop; sampling time 70 ms; modulation amplitude 10 mV.

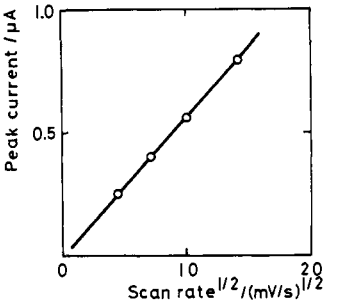

Figure 7. Relation between the peak current and the scan rate in cyclic voltammetry of ferricytochrome c_3: 5.31×10^{-5} M cytochrome c_3; electrode area 4.11×10^{-2} cm^2.

Cyclic Voltammetry. In the previous work we mentioned that the cyclic voltammetric peak of cytochrome c_3 was distorted and broader than expected from the one-electron electrode reaction.[22] That is, the broadening was much larger than that of the four-electron electrode reaction which is expected for the molecule with four strongly interacting hemes. The potentials at the peak currents for ferri- and ferrocytochrome c_3 did not shift on the potential axis over the scan rate from 20 to 200 mV s^{-1}. The ratio of the cathodic to anodic peak currents was almost unity. The plot of the peak current against the square root of the scan rate is linear and passes through the origin as shown in Figure 7. These results also suggested that the electrode reaction of the ferri-/ferrocytochrome c_3 couple is reversible through the adsorbed layer of the cytochrome c_3 molecule. The surface of the hanging mercury drop electrode is considered to be fully covered by cytochrome c_3 molecule in the present cyclic voltammetric measurements as is noted from the differential capacity measurement. The double layer capacitance at the mercury-cytochrome c_3 solution interface was about 9 μF cm^{-2} at -0.5 V in the present measurement. This value accords with that of the fully covered mercury electrode by cytochrome c.[31,32]

The measured cathodic peak current was only 1/2.5 of the current expected from a four-electron reversible electrode process with the diffusion coefficient of 0.94×10^{-6} cm^2 s^{-1}, which was obtained from the pulse polarographic data in the present experiments. The difference between the peak potential and the half-peak potential, $\Delta E_p = E_{p/2} - E_p$, was 0.073 V. The apparent number of electrons, n_{app}, was calculated to be 0.77.

Discussion

Pulse polarographic, scan-reversal pulse polarographic and linear potential-sweep voltammetric results indicate that the electrode reaction of ferri- and ferrocytochrome c_3 of *D. vulgaris*, strain Miyazaki, at mercury electrode is reversible and controlled by the diffusion of the protein molecule from the bulk of the solution to the electrode surface. The diffusion coefficient of ferricytochrome c_3 determined in the present work is 0.94×10^{-6} cm^2 s^{-1} and is in accordance with that of horse-heart ferricytochrome c (mol wt 12 350) determined by hydrodynamic technique, 0.95×10^{-6} cm^2 s^{-1}.[28] The electron transfer reaction rate of cytochrome c_3 is too fast to be determined by the present techniques and the rate constant is presumably on the order of 0.1 cm s^{-1} or greater. This is one of the fastest electron-transfer reactions at mercury electrode in aqueous solution.

The mass transport of cytochrome c_3 molecule from the bulk of the solution to the electrode surface which is covered by the adsorbed cytochrome c_3 molecule is the rate-determining step. The adsorption of cytochrome c_3 on the electrode surface is so strong that the displacement of the reduced form at the electrode by the oxidized form from the bulk of the solution is implausible. It was interesting to note that the charge transfer reaction of the ferri- and ferrocytochrome c_3 couple takes place through the strongly adsorbed layer of cytochrome c_3 on the dropping mercury electrode in spite of the slow electron exchange interaction among the hemes in the molecules. Since both ferro- and ferricytochrome c_3 are in the low-spin state,[19] the electron transfer between the heme edge adsorbed on the electrode and cytochrome c_3 molecule from the bulk of the solution is expected to be very fast as is observed in the present electrochemical measurements because the electron exchange between low-spin ferro- and ferrihemes in homogeneous phase is reported to be very fast.[29]

The absorption,[9] NMR,[14] and Mössbauer[19] spectroscopic measurements reveal that the separation between the hemes in cytochrome c_3 is 10 Å and that the electron exchange between the hemes is too slow to cause exchange averaging. In the electrochemical reaction of cytochrome c_3 the electron transfer between the hemes and the electrode would be inhibited by the protein fabric if proteins were adsorbed preferentially on the electrode.

The electrode reaction of horse-heart cytochrome c at mercury electrode is irreversible polarographically and the half-wave potential shifts in the cathodic direction with the increase of ferricytochrome c concentration.[30,31] The diffusion coefficient calculated from the polarographic limiting current is significantly lower than the value determined by hydrodynamic technique.[28] Betso and his co-workers[30] explained the reduction mechanism of cytochrome c in terms of either the compact globular structure of the protein which minimizes adsorption and faciliates displacement of reduced molecules by incoming oxidized ones, or by electron transfer through the surface layer which makes conduction or rotational diffusion possible. Scheller et al.[32] assumed that the electron-transfer mechanism of cytochrome c at the electrode is considered to be a superposition of electron transfer through the heme edge adsorbed on the electrode and displacement of the reduced proteins at the electrode surface by the oxidized proteins in the solution.

The most interesting feature of cytochrome c_3 is that the hemes in the protein are nonequivalent and noninteracting electrochemically and that the electrode reaction of these hemes is reversible through the adsorbed layer of cytochrome c_3 molecule on mercury electrode. A multistep very fast electron transfer reaction has to be postulated to explain pulse polarographic, differential pulse polarographic, potentiometric, and voltammetric results.

The overall electrode reaction of cytochrome c_3 is expressed as

$$\text{ferricytochrome } c_3 + 4e \rightleftharpoons \text{ferrocytochrome } c_3$$

A large Nernst slope, 93 mV, in the potentiometric measurement can be explained by assuming that the electrode reaction is four one-electron steps.[33] The equally spaced four standard potentials with −40 mV spacing generates a Nernst slope of about 90 mV over the c_0/c_R values from 0.2 to 5.0. A two two-electron step electrode reaction with the 50-mV spaced standard potentials was proposed for cytochrome c_3, strain Hildenborough, by Dobson et al.[15] The apparent Nernst slope generated from this value is about 50 mV over the c_0/c_R values from 0.2 to 5.0 but the deviation from the linear plot is appreciable. The ratio of ferro- to ferrihemes in the cytochrome c_3 of Hildenborough solution and the equilibrium potential of this system were measured simultaneously by DerVartanian et al.[34] In this case the ratio of ferri to ferro form was measured by ESR and the equilibrium potential at platinum electrode with a mediator. They predicted the four one-electron step electrode reaction of cytochrome c_3 with unequally spaced standard potential (the spacings are −26, −9, and −5 mV, respectively). When we replot their data, as we did in the potentiometry, the apparent Nernst slope becomes 81-91 mV, which is in good agreement with our present data of 93 mV, and the formal potential was estimated to be −0.32 V (vs. NHE).

The formal potential of the ferri- and ferrocytochrome c_3 couple is the average of the standard potentials of four hemes in the molecule. The formal potential determined in the present measurement agrees with our previous value, −0.53 V, from the equilibrium pressure of hydrogen over cytochrome c_3 and is different from the other measured value, −0.451 V.[4,5,7] The formal potential of −0.516 V obtained in the previous electrochemical measurement on mercury pool electrode[22] is slightly more positive than the present value of −0.528 V. This is probably due to the slow equilibrium of the redox couple at the surface of the coagulated mercury drops on the mercury pool.

In the cyclic voltammograms of cytochrome c_3 the ratio of the measured cathodic peak current to the calculated peak current for the reversible four-electron electrode process is 1/2.54. The peak broadening, $\Delta E_p = E_{p/2} - E_p$, of the cathodic peak was 73 mV and independent of the scan rate. This value is much larger than that expected from the reversible four-electron process, $\Delta E_p = 56.5/n$ mV, where n is the number of electrons involved in the charge-transfer process.

Evans and his co-workers[35] prepared a computer simulation by assuming that the electrode processes of cytochrome c_3 are four one-electron processes with equally spaced standard potentials of the four nonequivalent hemes. The ΔE_p value of 73 mV leads to the spacing between the standard potentials of −35 mV. This value is in good agreement with the spacing between the standard potentials to generate the Nernst slope of 93 mV in the potentiometry. The ratio of the peak current for the four one-electron processes with −35 mV spacing to the peak current for the four-electron process is calculated to be 1/2.23, which is also in good agreement with our experimental value of 1/2.54.

The simulations were made for the cases where the standard potentials of four hemes were spaced unequally.[35] The spacing varied from the ratio of 1:1:1 up to 5:2:5 but total potential spread was maintained constant. The simulated voltammograms were almost the same and Nernst slopes in the potentiometric titration generated from these standard potentials were also almost the same. The potentiometric and voltammetric results lead to the conclusions that the standard potentials of the four hemes in cytochrome c_3 are nonequivalent and the total potential spreading of the standard potentials is about 120 mV.

We found that the simulations on a slightly distorted differential pulse polarogram give us more information than that on cyclic voltammetry.[35] The differential pulse polarogram simulated from a model with unequally spaced standard potentials (about −50, −20, and −40 mV) is in good agreement with the observed one. The potentiometric data and voltammogram simulated with the above spacings also agree well with the observed values.

Although the amino acid sequence of cytochrome c_3 of *D. vulgaris*, strain Miyazaki, has not been established, it is reasonable to assume that the amino acid sequence of our cytochrome c_3 is similar to that of cytochrome c_3 of *D. vulgaris*, strain Hildenborough, because the amino acid compositions of both cytochrome c_3 are very similar.[9] That is, there are two types of hemes in cytochrome c_3 molecule and the binding groups to hemes are -Cys-a-b-Cys-His- and -Cys-a-b-c-d-Cys-His-, where a, b, c, and d represent amino acids.[15]

The sequence of the charge-transfer step from the mercury electrode to the hemes in cytochrome c_3 molecule is still uncertain from the present results. It is, however, reasonable to assume that the charge-transfer sequence is A, B, A′, and then B′ for a A_2B_2 type molecule as is stated for cytochrome c oxidase.[36]

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Isoprene Synthons. Silicon- and Tin-Mediated Terpene Synthesis[1]

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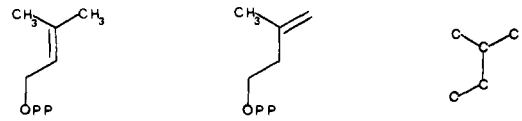
Abstract: Methodology for the construction of terpenes via reactive five-carbon "isoprene synthons" and applications of this strategy to the synthesis of various naturally occurring substances are discussed. The generation and chemistry of 1-cyclobutenylmethyllithium (7) and its conversion to 2-trimethylsilylmethylenecyclobutane (**10a**), trimethylsilylisoprene (**19a**), and trimethylstannylisoprene (**19b**) are described. Thermolysis of **10a**/**10b** leads to allylic isomerization followed by cyclobutene ring opening to **19a**/**19b**. Compounds **7**, **10a**, **19a**, and **19b** are useful terpene synthons. Compound **10a** reacts with isovaleraldehyde/$TiCl_4$ to yield cyclobutene **18** which can be thermolyzed to tagetol (**17**). Dienes **19a** and **19b** are used in a Diels–Alder reaction with methyl acrylate leading to a synthesis of δ-terpinol **22b**.

A terpene is a compound whose carbon skeleton is either directly constructed of isoprene units or has had at some state of its biosynthesis a carbon skeleton so constructed. The great majority of terpenes can thus be regarded as built up from the head-to-tail union of isoprene residues. Although methods for the head-to-tail linking of isoprene units have been the objective of much research in organic synthesis, the original isoprene synthon is used by nature herself. Dimethylallyl pyrophosphate (**1**) and isopentenyl pyrophosphate (**2**) condense to form ger-

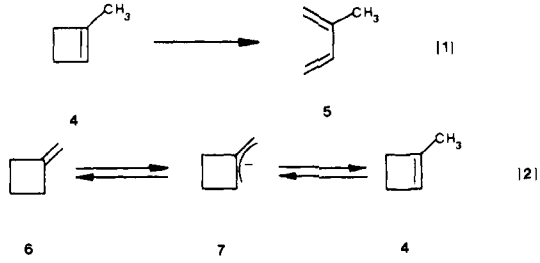


1 2 3

anyl pyrophosphate in the initial stages of the biosynthesis of many terpenes.[2]

Biogenetic-type[3] syntheses are basically synthetic routes designed to mimic as closely as possible steps in the biosynthesis. The use of isoprene synthons in terpene construction is not necessarily "biogenetic", although the regularity with which the repeating unit **3** appears in a large number of important compounds from nature means that 5-carbon *reagents* with the connectivity of **3** find wide applicability.

1-Cyclobutenylmethyllithium. Investigations of cyclobutene derivatives as reactive synthons for the introduction of isoprene residues have recently occupied our attention.[4] The strain energy of such small rings provides a strong driving force for chemical reactions. Thus, thermolysis[5] of 1-methylcyclobutene (**4**) to isoprene (**5**) (eq 1) is a well-known example of a con-

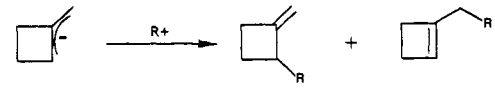


4 5 [1]

6 7 4 [2]

certed electrocyclic process. Simple generation of families of 1-substituted cyclobutenes is clearly a major objective if they are to become useful isoprene synthons. Therefore, the intermediacy of **7** in the reported[6] base-catalyzed isomerization (eq 2) of methylenecyclobutane (**6**) gave us the suggestion that the *quantitative* generation of **7** would yield an intermediate of unique applicability in this connection.

We found[4a] that metalation of methylenecyclobutane (**6**) with the *n*-butyllithium/TMEDA complex[7,8] in hexane occurred smoothly to give **7**.[9] Reaction of **7** with a proton was studied by D_2O quench. ^2H NMR (Figure 1) of the reaction mixture showed an encouraging 67% deuteration of the methylene group. CO_2 likewise reacts preponderantly at the methylene group as evidenced by the ratio of characteristic NMR signals at δ 4.8 (methylene) and 5.75 (cyclobutene H). However, the regioisomer problems typical of allyl anions[10,11] are clearly seen in the reactions of many other electrophiles (Table I). The **8**/**9** ratio was readily determined by NMR integration of vinyl proton signals at ca. δ 4.8 and the



7 8 9

results shown in the tables. A trend at least can be seen in the studies using $PhCH_2X$ and TMSX shown in Tables II and III. By varying the solvent and substrate it is possible to modify[12] the proportions of products within wide limits.[13] Higher temperatures and more reactive E^+ tend to favor reaction on the methylene yielding cyclobutene **9** whereas less reactive electrophiles and lower temperatures favor reaction on the ring to give **8**.

Mechanistic pathways to products **8** and **9** are shown in Scheme I. Because of the presence of TMEDA-complexed organolithium species, ion pairs, and aggregates, regioisomer control in the reactions of **7** proved fruitless.

Allylsilanes. We now describe the salient features of a silicon/tin based methodology which solves the regioisomer problems and allows additional flexibility in the incorporation of C-5 isoprene units. The unique reactivity of allylsilanes[15] has proved them to be functional groups with considerable

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