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Iron-histidine stretching Raman line and enzymic activities of bovine and bacterial cytochrome *c* oxidases

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Resonance Raman spectra of the reduced form of cytochrome *c* oxidase isolated from bovine heart and the thermophilic bacterium PS3 were investigated in relation to their H^+-pumping- and cytochrome-*c*-oxidizing activities, which were varied by incubating the enzyme at raised temperatures or at alkaline pH at room temperature. For both the bovine and PS3 enzymes, the intensity of the iron-histidine stretching Raman line of the ferrous a_3 heme (214 cm^{-1}) exhibited an incubation-temperature-dependent change, which fell between the similar curves of the H^+-pumping and cytochrome-*c*-oxidizing activities. The intensities of the formyl CH=O stretching Raman line of the ferrous a_3 heme (1665 cm^{-1}) as well as of other lines were insensitive to the heat treatment. The iron-histidine stretching Raman line of both enzymes showed pH-dependent intensity change which was nearly parallel with the pH dependence of cytochrome-*c*-oxidizing activity. Therefore, deprotonation affecting the 214 cm^{-1} Raman line is responsible for the decrease of activity. This limited alkaline treatment to the PS3 enzyme was reversible and the recovered enzyme exhibited Raman intensities and enzymic activities similar to the native one. However, the neutralized, bovine enzyme with a similar intensity of the 214 cm^{-1} line showed increased cytochrome-*c*-oxidizing activity and null H^+-pumping activity.

Introduction

Mitochondrial cytochrome *c* oxidase (cytochrome *c*, oxygen oxidoreductase, EC.1.9.3.1) contains two heme *a* groups and two copper atoms in more than seven subunits [1]. The low-spin and high-spin hemes in the resting state are designated as the *a* and a_3 heme, respectively. Recent studies revealed that bovine cytochrome *c* oxidase pumps protons across the membrane concurrently with its redox change [2,3]. Very similar enzymes, which have the same prosthetic groups but are composed of only two or three subunits, have been found and purified from several aerobic bacteria [4,5]. The bacterial cytochrome *c* oxidase isolated from thermophilic bacterium PS3 and *Thermus thermophilus* HB8 were demonstrated to possess reasonable H^+ pumping activities, when they were reconstituted into liposomes [6–10]. However, it remains to be elucidated how the vectorial H^+ translocation couples with the electron transfer.

Resonance Raman spectroscopy provides detailed structural information about the heme and its vicinity. Fundamental data on resonance Raman spectra of cytochrome *c* oxidase have already been reported [11–16]. The formyl CH=O stretching mode of the *a* heme was discussed in relation

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Abbreviations: Mops, 4-morpholinepropanesulfonic acid; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; Cyt, cytochrome.

to the H^+ pumping activity by Babcock and Callahan [17]. We note previously [18] that the intensity of the 213 cm^{-1} line of the PS3 enzyme in the reduced state changed largely when the H^+ pumping activity was altered; the PS3 enzyme incubated at 55–60°C exhibited significant reduction of the H^+ pumping activity without reduction of the cytochrome *c* oxidizing activity, and in its resonance Raman spectrum only the intensity of the 213 cm^{-1} line decreased appreciably. This Raman line was assigned to the iron-histidine (Fe-His) stretching vibration of the five coordinate ferrous high-spin a_3 heme on the basis of the ^{54}Fe isotopic frequency shift [19].

The similar differential effects on the two kinds of enzymic activities were found for heat treatment of bovine cytochrome *c* oxidase [20]. Therefore, in this study, we examined the effect of heat treatment on the intensity of the Fe-His stretching Raman line for bovine cytochrome *c* oxidase. In addition, we report the pH profiles of the iron-histidine stretching Raman intensities of the bovine and PS3 enzymes and their relevance to the H^+-pumping and cytochrome-*c*-oxidizing activities.

Materials and Methods

Two kinds of preparations of bovine cytochrome *c* oxidase [21,22] were kindly given by Dr. Orii of Kyoto University and Dr. Yoshikawa of Konan University. Bacterial cytochrome *c* oxidase was prepared from the thermophilic bacterium PS3 according to the procedure described previously [23]. Yeast cytochrome *c* from *Candida krusei* was a product of Sankyo Co., Tokyo.

For heat treatment the bovine enzyme with a concentration of 0.03–0.05 mM (as heme *a* content) was incubated in 10 mM phosphate buffer (pH 7.4) containing 0.3–0.5 mg/ml of cholate at the indicated temperature for 30 min and then brought into an ice bath. For alkaline treatment, pH of the bovine enzyme (0.05 mM) or the PS3 enzyme (0.03 mM) in 20 mM Tricine-NaOH containing 0.2% (w/v) octyl-glucoside was adjusted with a small aliquot of 0.01–0.5 M NaOH, and the pH value was determined after the Raman measurements (approx. 30 min).

For Raman experiments of the heat-treated enzyme, Triton X-100 was added to make its final concentration 0.5% (w/v), but the alkaline-treated enzyme was used as it was. An aliquot (50 μl) of the treated enzyme was put in a cylindrical cell, reduced with a grain of $Na_2S_2O_4$, and then evacuated to 1.33 Pa. Raman spectra were measured at 5°C with the 441.6 nm line from He/Cd laser (Kinmon Electrics, Model CDR80MGE), and recorded with a JEOL-400D Raman spectrometer equipped with a cooled RCA31034*a* photomultiplier. Intensity of a Raman line was evaluated as peak-height from a reasonable base line.

Although all the heat-treated samples for Raman measurements contained 7.0% (w/v) of $(NH_4)_2SO_4$, the intensities of Raman lines are represented as relative intensity to the ν_4 mode at 1357 cm^{-1}, but not to the sulfate line at 981 cm^{-1} due to the following reason; during incubation at raised pH or temperature some part of the enzyme precipitated and therefore the effective concentration of the enzyme was a little altered in each measurement. In fact, as will be shown in Fig. 1 later, when the relative intensity to the sulfate line is used for the enzyme incubated at 43.5°C for 30 min, intensities of all Raman lines decreased to 60–90%, but the intensity of the 214 cm^{-1} line decreased to 38% in comparison with those of non-incubated enzyme. It is desirable to cancel the precipitation effect in the intensity representation. Since the ν_4 mode gives the most intense Raman line and its intensity seems to involve less error, we adopted this line as a standard, although it is optional.

For the measurement of cytochrome *c* oxidizing and H^+ pumping activities, another aliquot (10 μl for the bovine enzyme and 25 μl for the PS3 enzyme) of the treated enzyme was reconstituted into proteoliposomes with 0.45 ml of acetone-washed α-tocopherol-treated soybean phospholipids [24] by the freeze-thaw-sonication method as described previously [10]. The liposome solution (75 μl) thus prepared was put into the reaction medium (1.6 ml) composed of 25 mM K_2SO_4/2.5 mM $MgSO_4$/0.25 mM K–Mops buffer (pH 7.0), and the amount of the pumped protons was measured with a pH meter (Beckman model 4500 with a combination electrode, Beckman 39505) at 30°C for bovine enzyme and at 33°C for the PS3 enzyme as described previously [20].

The H^+ pumping activity was evaluated from

the initial rate of H^+ extrusion upon addition of 4–5 nmol *Candida* cytochrome *c*. Cytochrome *c* oxidizing activity was regarded as the initial rate of H^+ uptake upon addition of cytochrome *c* to the reaction medium in the presence of ferrocyanide (2 μmol), valinomycin (50 ng) and FCCP (250 ng). This activity was proportional to that obtained by a usual assay of cytochrome *c* oxidizing activity with an oxygen electrode [25]. In this paper the proton pumping activity is represented in terms of H^+/e^- ratio, i.e., the initial rate of H^+ extrusion in the absence of FCCP divided by that of H^+ uptake in the presence of FCCP [20].

Results

Effects of heat treatment on the bovine enzyme

Incubation of bovine cytochrome *c* oxidase at 43–48°C results in selective inactivation of the H^+ pumping activity without diminution of cytochrome *c* oxidizing activity [20]. Fig. 1 compares the resonance Raman spectrum of the reduced form of Yoshikawa's preparation [22] incubated at 43.5°C for 30 min (B) with that of the native reduced form (A). The two spectra are very alike except for noticeable intensity reduction of the Raman line at 214 cm^{-1} in spectrum B. Qualitatively the similar phenomenon was observed for Orii's preparation [21]. The Raman line at 214 cm^{-1} disappeared upon binding of carbonmonoxide and cyanide, and was therefore assigned to the iron-histidine stretching mode of the a_3 heme [19]. This spectral change was similar to what occurred in the PS3 enzyme incubated at 60°C for 20 min [18].

For evaluation of Raman intensities, an appropriate base line shown by a broken line in Fig. 1 was assumed and the peak-height from this line was measured. The maximum intensities of Raman lines at 214 and 1665 cm^{-1} relative to that at 1357 cm^{-1}, I_{214}/I_{1357} and I_{1665}/I_{1357}, of the reduced form of the heat-treated bovine enzyme from Orii's preparation are plotted against the incubation temperature in Fig. 2, where the H^+ pumping and cytochrome *c* oxidizing activities of the enzymes used for the Raman measurements are also plotted. The H^+ pumping activity remained unaltered up to 38°C but decreased drastically at 45°C. Cytochrome *c* oxidizing activity increased gradually up to 48°C and then decreased to zero with the midpoint temperature at 52°C. The temperature dependence of intensity of

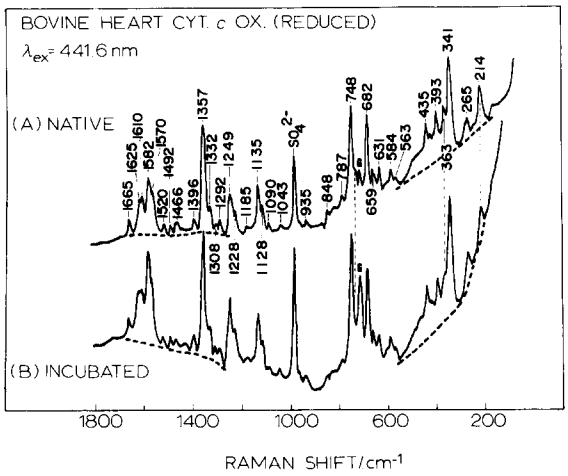

Fig. 1. Resonance Raman spectra of the reduced form of bovine heart cytochrome *c* oxidase from Yoshikawa's preparation. (A) native state; (B) incubated at 43.5°C for 30 min and quenched in the ice bath. 'G' denotes a grating ghost. The Raman line marked with SO_4^{2-} is due to $(NH_4)_2SO_4$ present (7%) in the buffer as an intensity standard. Instrumental conditions: scan speed, 50 cm^{-1}/min; sensitivity, 25000 counts/s; time constant, 0.4 s; spectral slit width, 7 cm^{-1}; laser power, 40 mW.

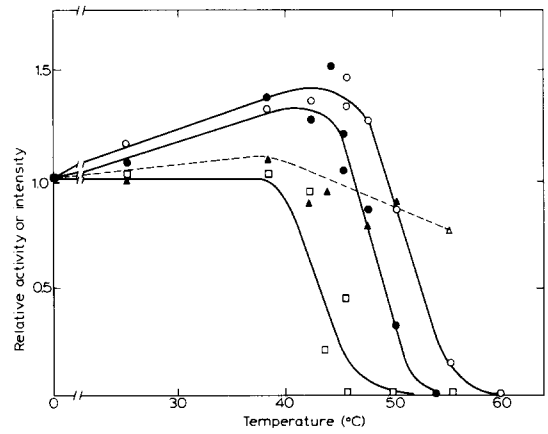

Fig. 2. Incubation temperature dependences of the relative Raman intensities of the heat-treated bovine enzyme of Orii's preparation and their enzymic activities; I_{214}/I_{1357} (●); I_{1665}/I_{1357} (▲); H^+-pumping activity designated by the H^+/e^- ratio (□); cytochrome *c* oxidizing activity (○). The ordinate stands for relative values to the corresponding values obtained for the enzyme preincubated at 0°C. The latter values are; $I_{214}/I_{1357} = 0.21$, $I_{1665}/I_{1357} = 0.15$, $H^+/e^- = 1.2$, and cytochrome *c* oxidizing activity = 13.1 nmol/min.

the 214 cm^{-1} line fell between the similar curves of two kinds of enzymic activities as was so for the PS3 enzyme [18]. The intensity of the Raman line at 1665 cm^{-1}, which is known to arise from the CH=O stretching mode of the peripheral formyl group of the a_3 heme [18,26,27], was scarcely changed up to 55°C, above which the sample became seriously turbid and its resonance Raman spectrum could not be observed satisfactorily. It is noted that the absolute values of the Raman relative intensities were appreciably different between the two preparations due to unknown reasons, but their temperature dependences were alike. Thus, the trend shown in Fig. 2 is independent from the preparation method.

pH-dependent changes of the bovine enzyme

It was previously reported by Callahan and Babcock [28] that alkalization of the enzyme in the reduced form caused intensity reduction of the 1665-cm^{-1} line with a midpoint pH at 9.3, and at the extremely alkaline conditions such as at pH 12 a further change took place probably due to Schiff base formation of the formyl group. However, a systematic examination about possible relationships between the resonance Raman spectra and enzymic activities has never been investigated so far.

Fig. 3 illustrates the pH dependent intensity change of the iron-histidine and the formyl CH=O stretching Raman lines of the bovine enzyme. Intensity reduction of the iron-histidine stretching Raman line at 214 cm^{-1} occurred with a midpoint pH at 8.6 under the present solution conditions, while the CH=O stretching line (1665 cm^{-1}) changed with a midpoint pH at 8.9. The pH profile of cytochrome-*c*-oxidizing activity is also included in Fig. 3. A part of this curve (above pH 8) bears some resemblance to the pH profile of I_{214}/I_{1357}, suggesting that deprotonation of a residue which affects the iron-histidine stretching Raman intensity also affects the oxidase activity. Difference between the overall pH profiles obtained from the Raman intensity and the oxidase activity is partly due to experimental errors and partly due to another effect of pH on the oxidase activity, that is, on the affinity for cytochrome *c* binding. The affinity primarily depends on the surface residues and their protonation/deprotonation may be superimposed on the pH-dependent profile of the activity of dioxygen reduction but not of the Raman intensity. The oxidase activity was less pH-dependent between pH 6.2 and 8.0 with higher concentration of cytochrome *c*. The H^+/e^- ratio was 1.0–1.2 at pH values between 6.2 and 8.4 above which it could not be measured, and thus seemed to be unaffected by the pH change.

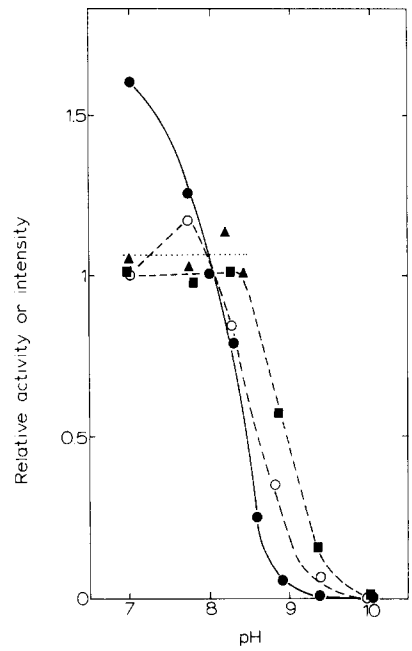

Fig. 3. pH dependent changes in relative resonance Raman intensities and enzymic activity of bovine cytochrome *c* oxidase; I_{214}/I_{1357} (○), I_{1665}/I_{1357} (■), and those in cytochrome *c* oxidizing (●) and H^+ pumping (▲) activities. Raman plots are normalized by the values at pH 7, which are actually 0.21, and 0.14 for I_{214}/I_{1357}, I_{1665}/I_{1357}, respectively. Enzymic activities are normalized by the values at pH 8, but actually cytochrome *c* oxidizing activity at pH 8 is 15.7 nmol/min and the H^+/e^- ratio is 1.0.

pH dependent changes of the PS3 enzyme

The cytochrome *c* oxidizing and H^+ pumping activities of the PS3 enzyme are plotted against pH in Fig. 4, where pH dependence of the iron-histidine stretching Raman intensity of the PS3 enzyme used for the activity measurements are also included. The solid line denotes an expected curve calculated for ionization of a single residue with $pK_a = 8.6$. Although points are somewhat

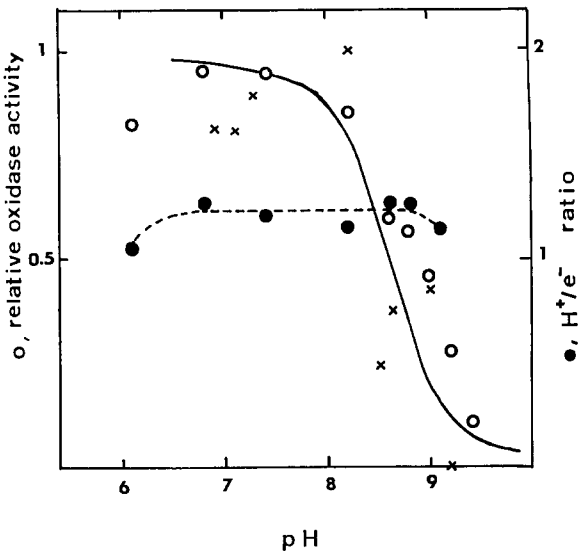

Fig. 4. pH dependence of cytochrome *c* oxidizing (○) and H^+-pumping (●, designated in terms of H^+/e^- ratio) activities and of relative resonance Raman intensity (×, I_{213}/I_{1356}) of the PS3 enzyme. The solid line denotes an expected curve calculated for ionization of a single residue with $pK_a = 8.6$.

scattered, the pH profile of the Raman intensity appears qualitatively parallel with that of the cytochrome *c* oxidizing activity, suggesting that the protonated form of the particular amino acid residue in the PS3 enzyme, which leads to appearance of the Raman line at 213 cm^{-1}, is associated with the oxidase activity. On the other hand, the H^+-pumping activity represented in terms of the H^+/e^- ratio was again unaffected by changing pH from 6.1 to 9.1, above which it could not be measured.

Reversibility of alkaline treatment

The pH-dependent changes of Raman intensity and oxidase activity described above were reversible unless the protein was exposed to extremely alkaline pH. The recovery of the alkaline-treated enzyme upon neutralization to pH 7 is summarized in Table I. In the case of PS3 enzyme almost full recovery was confirmed up to pH 10.1 about the three properties examined. The treatment at pH 10.4 made an appreciable portion of the changes irreversible. With regard to the bovine enzyme, on the other hand, the treatment at pH 9.6 for 3 h exhibited 90% recovery for the Raman

TABLE I

REVERSIBILITY FROM ALKALINE TREATMENT

As for the $I_{Fe\text{-}N}/I_{1357}$ ratios $\nu_{Fe\text{-}N} = 213$ cm^{-1} for PS3 enzyme and $\nu_{Fe\text{-}N} = 214$ cm^{-1} for bovine enzyme.

Enzyme	Alkalization		Recovery after neutralization		
	pH	hours	$I_{Fe\text{-}N}/I_{1357}$	oxidation (μmol/min)	H^+/e^-
PS3	control		0.09	5.0	1.1
	10.1	3	0.11	5.0	1.1
	10.4	1/3	0.08	4.6	0.84
Bovine	control		0.23	21.5	1.2
	9.6	3	0.21	26.0	0
	10.3	3	0.16	22.6	0
	10.3	13	0	-	-

intensity and 21% increase for cytochrome *c* oxidizing activity but null for the H^+-pumping activity. The excess recovery of the cytochrome *c* oxidizing activity did confirm the reported 'alkaline activation' [29], which is considered to be caused by conversion from a dimer to monomers at an alkaline pH [30,31].

Discussion

The 441.6-nm excitation of Raman scattering from reduced cytochrome *c* oxidase reveals mainly the contribution from the cytochrome *a* moiety [32,33] with a few exceptional lines, that is, the iron-histidine and formyl-stretching modes, for which Raman lines from cytochrome a_3 are clearly observed. Therefore, except for the two lines, the 441.6-nm-excited resonance Raman spectrum of reduced cytochrome *c* oxidase is apparently unaltered upon binding of a ligand to cytochrome a_3 [33].

The iron-histidine stretching Raman line of the reduced a_3 heme showed interesting responses to alkalization and heat denaturation. The intensity of this line is roughly proportional to the number of five-coordinated ferrous high-spin a_3 hemes [19]. Therefore, the intensity reduction of the iron-histidine stretching Raman line implies that coordination of some ligand to the sixth coordination site of the ferrous a_3 heme, and this is likely to be triggered by deprotonation of a specific residue having its pK_a near 8.6, possibly distal

histidine as in other heme proteins [34]. Reasonable correspondence between the pH profiles of intensity of the Fe-histidine stretching Raman line and cytochrome *c* oxidizing activity may suggest that the proton of distal histidine plays an important role for the reduction of dioxygen to water similar to the case of horseradish peroxidase [35].

It was also shown that the H^+/e^- ratio was not pH-dependent in the case of the limited alkalization (Figs. 3 and 4). This means that, although the number of active enzymes with regard to the cytochrome *c* oxidizing activity decreases with increase of pH, the active enzymes pump H^+ quite normally as in a neutral solution. The PS3 enzyme which was restored from the alkaline treatment exhibited the same H^+-pumping activity as the original enzyme, but the bovine enzyme neutralized after alkaline treatment (pH 9.6), did not pump H^+ in spite that the 214 cm^{-1} Raman line was almost recovered (Table I). The reason of this difference between the two enzymes is not known, but may possibly be related to the fact that the bovine enzyme behaved as a monomer in a gel-filtration column after the alkaline treatment [31], while the PS3 enzyme kept its dimeric elution pattern (Sone, N., unpublished results). This fact supports the proposed hypothesis that only dimeric form of the enzyme, not a monomeric one, pumps H^+ [36]. In favor of this hypothesis, there is another observation that the target size deduced from high-energy electron-beam irradiation for the H^+-pumping activity is 2–3-times larger than that for the oxidase activity for both PS3 and bovine enzymes (Sone, N., and Kasako, T., unpublished data). Very recently, however, it was reported that a monomeric shark cytochrome *c* oxidase pumps H^+ [37], which is against that hypothesis. Thus, it is not decisive at the present stage, but if such dimer-monomer conversion model, in which one monomer component of the dimer has no cytochrome *c* oxidizing activity despite the similar structures in the heme vicinity to that of the active monomer, were accepted, then it would be likely that neither the iron-histidine stretching intensity nor absorption spectrum is altered upon monomerization.

The heat treatment of the bovine enzyme around 45°C produced the species having the normal cytochrome *c* oxidizing activity, but decreased H^+-pumping activity. We suggested previously the presence of some correlation between the intensity of the iron-histidine stretching Raman line of the ferrous a_3 heme and the H^+-pumping activity for the PS3 enzyme [18]. As expected, the intensity of the 214 cm^{-1} line of the heat-treated enzyme was much weaker than that of the native one for the bovine enzyme, too (Fig. 1). This was confirmed for two independent preparations of bovine enzymes. However, for the bovine enzyme, the incubation temperature dependence of the Raman intensity was rather different from that of the H^+-pumping activity (Fig. 2) and the ordinate scale for Raman intensity appreciably depended upon preparations. This may imply that the iron-histidine bond of cytochrome a_3 has no direct connection with proton pumping. Although this possibility cannot be ruled out, the results shown in Figs. 2–4 rather suggest the presence of some indirect connection. From the latter point of view, it is likely that a protein structure at the pumping site suffers some damage upon heat treatment, and this structural change is communicated to the vicinity of the a_3 heme, resulting in a change of the iron-histidine stretching mode. It is finally stressed that enzymes which give the normal 441.6-nm-excited resonance Raman spectra, but lack the iron-histidine stretching Raman line of the ferrous a_3 heme, do not perform the H^+ pump, and in this regard the iron-histidine stretching Raman line is a sensitive and interesting probe of the active site of cytochrome *c* oxidase.

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