# Effect of Heat Treatment on Oxidase Activity and Proton-Pumping Capability of Proteoliposome-Incorporated Beef Heart Cytochrome $aa_3$†

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ABSTRACT: By incubating beef heart cytochrome *c* oxidase at 43–45 °C, selective inactivation of the $H^+$-pumping function is possible without affecting cytochrome *c* oxidase activity; proteoliposomes reconstituted with heated enzyme (43.5 °C for 60 min at pH 7.0) showed an apparent $H^+/e^-$ ratio of only 0.3 and a turnover with cytochrome *c* plus ferrocyanide as substrate of 20 $s^{-1}$, while those with the intact enzyme showed an apparent $H^+/e^-$ ratio somewhat greater than 1.0 and a turnover of 19 $s^{-1}$. This decrease in the $H^+/e^-$ ratio could not be attributed to a stimulation of $H^+$ permeability upon heating, since the respiratory control ratio and the magnitude of membrane potential formation remained almost the same in the two cases. A pH-dependent $E_m$ (midpoint redox potential) change of cytochrome *a* in the presence of cyanide was still observed after the heat treatment. Heating induced a small spectral shift in the Soret region of the oxidized (resting) enzyme; the peak of the heated enzyme was at 421 nm, while that of the intact enzyme was at 419 nm. The spectral shift obtained by pulsing the enzyme with oxygen under turnover conditions is also altered.

Recent observations with purified cytochrome *c* oxidase reconstituted into liposomes have been interpreted as showing that the enzyme pumps protons in addition to simply carrying electrons across the membrane (Wikström & Saari, 1977; Sigel & Carafoli, 1978; Casey et al., 1979; Sone & Hinkle, 1982; Proteau et al., 1983). This proposed $H^+$-pumping activity of cytochrome *c* oxidase, however, is still controversial (Mitchell & Moyle, 1983; Papa et al., 1983). At least one of the reasons for this seems to be that some cytochrome *c* oxidase preparations actually do not induce $H^+$ movement although their oxidase activity is intact. Discrepancies are also observed among the recently purified bacterial cytochrome $aa_3$ oxidases; the enzymes from the thermophilic bacterium PS3 (Sone & Hinkle, 1982; Sone & Yanagita, 1984) and from *Thermus thermophilus* HB8 (Sone et al., 1983) apparently showed good $H^+$ pumping, while oxidases from *Rhodopseudomonas spheroides* (Gennis et al., 1982) and from *Nitrobacter agilis* (Sone et al., 1983) did not. In the case of *Paracoccus denitrificans*, $H^+$-pumping activity has now been reported (Solioz et al., 1982) after several initial failures (Ludwig, 1980).

Removal of subunit III of the beef heart enzyme is observed to result in a loss of $H^+$-pumping capability (Saraste et al., 1981; Penttilä, 1983). And *N,N'*-dicyclohexylcarbodiimide, a potent inhibitor of the $H^+$ channel portion ($F_0$) of $H^+$-ATP synthetase ($F_0F_1$-ATPase), also modifies subunit III with a concomitant loss of $H^+$-pumping activity (Casey et al., 1980; Prochaska et al., 1981).

We have found a differential sensitivity of the $H^+$-pumping activity and the oxidase activity of PS3 cytochrome oxidase toward heat denaturation; $H^+$-pumping activity is lost at 55–60 °C, while oxidase activity is not (Ogura et al., 1984), in accordance with the earlier report (Sone et al., 1979). Following this same moderate heat treatment, almost all the resonance Raman lines, including that at 1610 $cm^{-1}$ for the formyl vibration of ferrocytochrome *a*, which was in fact suggested by Babcock & Callahan (1983) to be implicated in $H^+$ pumping, are scarcely changed, but the 213-$cm^{-1}$ line, assigned to ferrous heme *a*–histidine stretching by Ogura et al. (1983), is diminished drastically (Ogura et al., 1984). Here we report the effects of differential heat denaturation on beef heart cytochrome oxidase.

## Materials and Methods

Beef heart cytochrome *c* oxidase was prepared by the method of Kuboyama et al. (1972), with Tween 80 substituting for Emasol, and stored at −75 °C as previously described (Nicholls & Hildebrandt, 1978). Cytochrome *c* type VI from horse heart, cytochrome *c* type VIII from *Saccharomyces cerevisiae*, FCCP,[1] valinomycin, and MOPS were products of Sigma, St. Louis, MO. Nigericin was obtained from Calbiochem.

Heat treatment of cytochrome oxidase was carried out by incubating the enzyme (10–20 μM as cytochrome $aa_3$) for 30–60 min in 10 mM K-MOPS buffer (pH 7.0) at 43–43.5 °C, unless otherwise described.

Vectorial reactions by cytochrome *c* oxidase were measured after reconstituting the enzyme into liposomes by a freeze–thaw method (Kasahara & Hinkle, 1976) as follows: 40 mg of acetone-washed α-tocopherol-treated soybean phospholipids (Sone et al., 1977) was suspended in 1 mL of 5 mM K-MOPS buffer (pH 6.6) containing 2.5 mM $K_2SO_4$ and 0.1 mM EDTA and homogenized sufficiently with a vortex mixer. Cytochrome *c* oxidase (0.3–1.5 nmol) was then added and mixed well. The mixture in the test tube was frozen by being placed in a −75 °C freezer and then after at least 30 min thawed at room temperature and subjected to a brief 10-s sonication in a bath-type sonicator (150-W type, Esterline Angus). The freeze–thaw–sonication cycle was repeated once more, but the final sonication was for only 5 s.

Proton-pumping activity was measured as the initial $H^+$ extrusion activity in the presence of valinomycin upon addition of ferrocyanide and *S. cerevisiae* cytochrome *c*. This initial rate obtained was divided by the initial rate of scalar $H^+$ uptake in the presence of the protonophore FCCP under the same conditions, in order to obtain the $H^+/e^-$ ratio (Sone &

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[1] Abbreviations: FCCP, carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone; MOPS, 4-morpholinepropanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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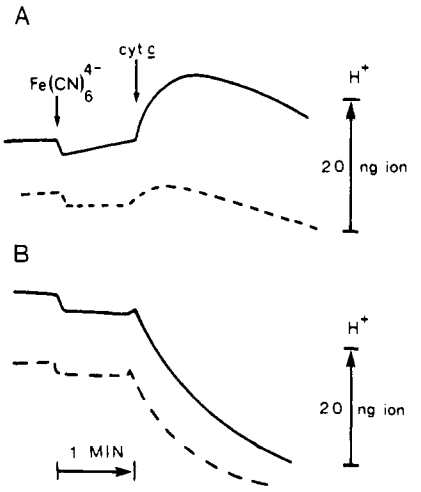


FIGURE 1: Measurement of $H^+$-pump activity and effect of heat treatment upon cytochrome $aa_3$. The reaction medium (2.5 mL) contained 25 mM $K_2SO_4$, 2.5 mM $MgSO_4$, and 0.25 mM K-MOPS buffer (pH 7.0). A 75-$\mu$L aliquot of proteoliposomes containing 3 mg of phospholipids and 0.026 nmol of cytochrome $aa_3$ was added together with valinomycin (0.2 $\mu$g) and incubated at 30 °C for about 10 min. The reaction was started by the addition of cytochrome *c* (*S. cerevisiae*, 4 nmol) and ferrocyanide (0.8 $\mu$mol) at the points indicated (↓). (A) Control without FCCP; (B) uncoupled with FCCP (1 $\mu$g); (—) proteoliposomes with intact enzyme; (– –) proteoliposomes with heated enzyme.

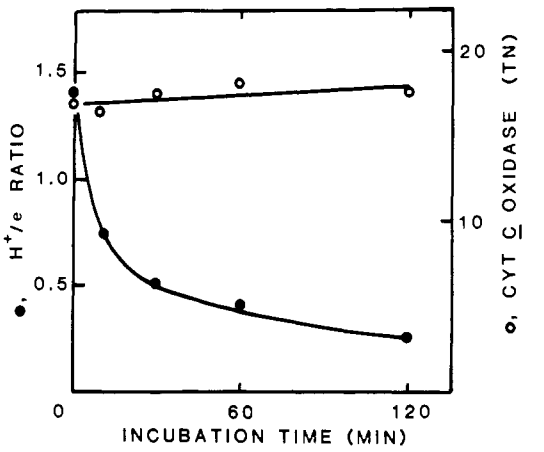


FIGURE 2: Effect of incubation time on $H^+$-pumping activity and an oxidase activity. The heat treatment was carried out at 43 °C in 10 mM K-MOPS buffer at pH 7.0. Reconstitution of the enzyme into proteoliposomes and the assay conditions for both $H^+$-pumping activity and oxidase activity were the same as those in Figure 1.

Yanagita, 1984). A Beckman pH meter ($\phi$ 71) with a combination electrode (Metrohm, semimicro type) was used, and the pH change was followed by a sensitive strip-chart recorder (Cole-Parmer, Model 8371-10).

Oxidase activity was measured as the scalar $H^+$ uptake in the presence of valinomycin and FCCP (in the case of proteoliposomes) or without any additions (soluble cytochrome oxidase in the presence of a detergent) with the pH meter as described above. Respiratory control, stimulation of respiration upon addition of FCCP, was measured with a Clark-type oxygen electrode (Yellow Springs Instrument Co.). Membrane potential formation was measured by using a hand-made $BTPP^+$ (butyltriphenylphosphonium chloride) electrode with a poly-vinyl chloride membrane containing tetraphenylboron (Kamo et al., 1979).

Absorption spectra were measured in a Beckman DU-7 spectrophotometer at a scanning rate of 10 nm/s, and the data acquired were stored on an on-line Apple II plus microcomputer (Chanady et al., 1984). The concentration of cytochrome $aa_3$ was determined from an extinction coefficient of 27 $mM^{-1}$ for the reduced minus oxidized spectrum, 605 nm – 630 nm, and that of horse heart cytochrome *c* from a value of 21.8 $mM^{-1}$ at 550 nm – 540 nm (Nicholls & Chanady, 1982). For the *S. cerevisiae* cytochrome *c* determination, an extinction coefficient (reduced minus oxidized) of 21.2 $mM^{-1}$ at 550 nm (Yonetani & Ray, 1965) was used.

## Results

*Effects of Heat Treatment on Proton Pumping.* Beef heart cytochrome *c* oxidase was incubated at 43.5 °C for 60 min as described under Materials and Methods and then reconstituted into proteoliposomes with soybean phospholipids. Figure 1A shows the pH change occurring in a reaction medium containing $K^+$ plus valinomycin upon oxidation of cytochrome *c* in the presence of ferrocyanide as a final electron donor. A decrease of pH, indicating vectorial $H^+$ ejection from liposomes containing the intact enzyme, was seen in the initial stage following cytochrome *c* addition (continuous trace). Then, this initial decrease in pH was gradually compensated by the net alkalinization that took place in the liposomal lumen. When liposomes with the heated enzyme were used (dashed trace), only a slow $H^+$ ejection was found. In the presence of FCCP (Figure 1B), the addition of cytochrome *c* resulted in an immediate alkalinization of the medium (scalar $H^+$ utilization) with liposomes either from the intact enzyme (continuous trace) or from the heated enzyme (dashed trace), indicating that the oxidase activities were similar. Since the initial rates of $H^+$ ejection (without FCCP) were 42 ng of ion/min for the intact enzyme and 9.5 ng of ion/min for the heated enzyme (Figure 1A and similar traces) and the initial rates of alkalinization in the presence of FCCP were 28 ng of ion/min for the intact enzyme and 27 ng of ion/min for the heated enzyme (Figure 1B and similar traces), the rate of oxidation does not seem to show appreciable "respiratory control" in the presence of valinomycin at least in the initial stages following the addition of a small amount of cytochrome *c*. In each such experiment, the $H^+/e^-$ ratio obtained for the intact enzyme was greater than or equal to 1.00, and that for the heated enzyme was no longer than 0.35. Values for the $H^+/e^-$ ratio exceeding 1.00 were also seen in the case of PS3 cytochrome oxidase (Sone & Yanagita, 1984) and are discussed below. Reduced cytochrome *c* pulses to an aerobic system containing valinomycin and higher vesicle concentrations gave analogous results, but with $H^+/e^-$ ratios below 1.0 for the intact enzyme (data not shown). Similar findings with PS3 oxidase are described and reported by Sone & Yanagita (1984). The reasons for the lower measured ratios with the pulse method are being investigated further (S. Shaughnessy et al., unpublished results).

Figure 2 shows a time course for the heat treatment at 43 °C. After 30-min incubation, the $H^+/e^-$ ratio decreased dramatically and fell below a value of 0.3 after 2-h incubation, while the oxidase activity was almost constant. Figure 3 shows the effect of incubation temperature on the $H^+/e^-$ ratio and on the oxidation rate. At 50 °C, cytochrome *c* oxidase activity itself was also lost. At 45 °C in this experiment, the oxidase activity seemed not to be injured, but occasionally the enzyme solution became turbid. For these reasons, we chose an incubation temperature at 43–43.5 °C for the subsequent experiments.

*Effects of Heat Treatment on Kinetics of Cytochrome c Oxidation.* Mitochondrial cytochrome *c* oxidases are known to have two cytochrome *c* binding sites, a high-affinity site and a low-affinity site (Nicholls, 1964; Ferguson-Miller et al.,

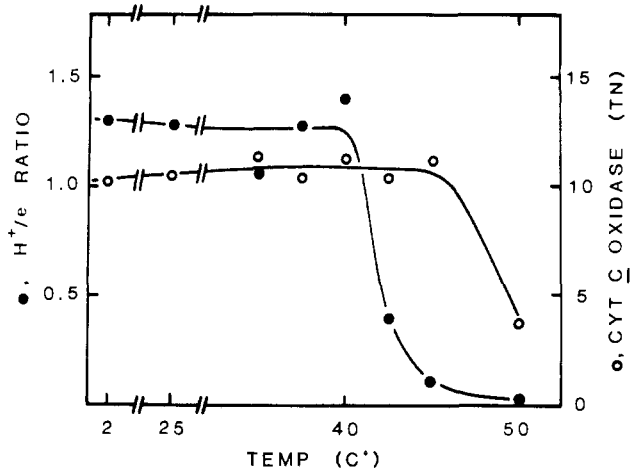


FIGURE 3: Differential sensitivity of $H^+$-pumping activity and oxidase activity toward heat denaturation. The heat treatment was carried out in 10 mM K-MOPS buffer, pH 7.0, at the indicated temperature for 30 min. Reconstitution of the enzyme into proteoliposomes and the assay conditions to measure $H^+$-pump activity (●) were the same as those for Figure 1. The cytochrome *c* oxidase activity (○) was measured by using 10 mM ascorbate as a substrate in the presence of 1.6 μM *S. cerevisiae* cytochrome *c* and 0.4 μg/mL FCCP and followed with a pH meter as shown in Figure 1B.

Table I: Effect of Heat Treatment of Cytochrome $aa_3$ upon Cytochrome *c* Oxidation Kinetics[a]

| | parameter measured | enzyme prepn: control | enzyme prepn: heat treated |
|---|---|---|---|
| high-affinity (T) site | $V_{max}$(app) ($s^{-1}$) | 120 | 150 |
| | $K_m$ (μM) | 0.16 | 0.23 |
| low-affinity (L) site | $V_{max}$ ($s^{-1}$) | 400 | 320 |
| | $K_m$ (μM) | 8.0 | 8.6 |

[a] Conditions are as described under Materials and Methods. Approximately 0.037 μM cytochrome $aa_3$ was used in the presence of varying concentrations of cytochrome *c* plus 10 mM sodium ascorbate and 0.15 mM TMPD in a medium containing 20 mM KCl, 1 mM K-MOPS buffer, and 0.2% Tween 80 at pH 6.8 and 30 °C.

1976). Since the experiments described above were carried out only at a low level of cytochrome *c* (1.6 μM), the effects of cytochrome *c* concentration on the oxidation rate were examined with the pH meter in the presence of 10 mM ascorbate and 0.15 mM TMPD. The ratio of velocity to substrate concentration ($v$/[s]) were plotted against the velocity ($v$). The results are summarized in Table I. For the high-affinity site, $V_{max}$ and $K_m$ were slightly increased by heat treatment whereas for the low-affinity site the $V_{max}$ declined slightly after treatment. The changes are of doubtful significance, and these results indicate that at most the heat treatment inhibits oxidase activity slightly, only when the electron flow is rapid.

*Effects on Respiratory Control and Membrane Potential Formation.* Proteoliposomes prepared as described under Materials and Methods with the heated enzyme show respiratory control when tested in the standard oxygen electrode system with ascorbate, TMPD, and *S. cerevisiae* cytochrome *c* as substrates. Respiratory control ratios for oxygen uptake rates in the presence and absence of FCCP of 4 or better were obtained, similar to the values obtained with vesicles containing intact enzyme. Figure 4 shows that the membrane potential as monitored by $BTPP^+$ uptake and formed by liposomes containing heated enzyme is also comparable to that formed by those containing intact enzyme. With some proteoliposome samples, membrane potential formation by liposomes with the heated enzyme was indeed slightly smaller than that by those

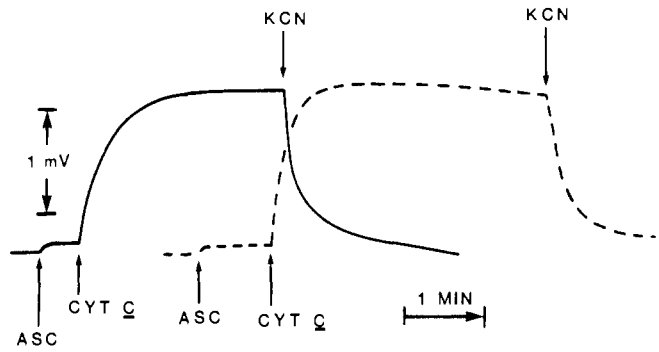


FIGURE 4: Membrane potentials formed by proteoliposomes reconstituted from the heat-treated enzyme and from the intact enzyme. The reaction was carried out in a reaction medium (2.5 mL) composed of 25 mM $K_2SO_4$, 2.5 mM $MgSO_4$, 5 μM $BTPP^+$, and 5 mM ($K^+$) phosphate buffer at pH 7.2. A 50-μL aliquot of proteoliposomes containing 0.03 nmol of cytochrome $aa_3$ was used. The membrane potential formation was followed by measuring $BTPP^+$ uptake with a $BTPP^+$ electrode at 30 °C. The additions are (ASC) 10 mM sodium ascorbate, (CYT *c*) 10 μM horse heart cytochrome *c*, and (KCN) 1 mM KCN. (—) Intact enzyme (control); (--) heat-treated enzyme.

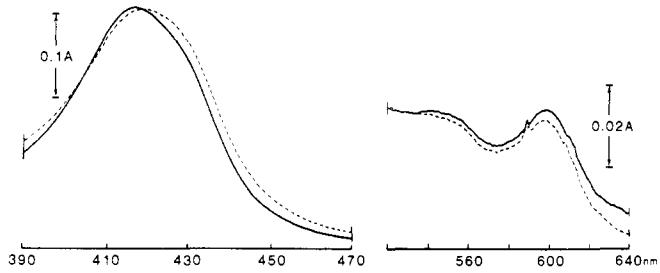


FIGURE 5: Effect of heat treatment on the spectrum of the resting form of cytochrome $aa_3$. The heat treatment was carried out in 10 mM ($K^+$) MOPS buffer, pH 7.0, at 43 °C for 40 min (--) or at 22 °C [control (—)]. A 0.2-mL aliquot of the enzyme solution was diluted with 0.8 mL of 50 mM sodium phosphate buffer (pH 7.4) containing 0.25% Tween 80. The absolute spectra for the oxidized (resting) states were recorded as shown.

with the intact enzyme, but the difference was always small. These data suggest that transmembrane electron flow is actively operating after heat treatment and that the decrease in $H^+$ ejection after the heat treatment is not caused by an increase in $H^+$ back-flow (a stimulation of $H^+$ permeability).

*Spectral Changes Induced by Heat Treatment.* Figure 5 shows that a small but characteristic shift is induced in the Soret region of the resting enzyme following heat treatment; the Soret peak moves from 419 to 421 nm (heated), and a small shoulder is found at 425 nm in the case of the heated enzyme. No significant change seems to occur in the α region. In the case of the fully reduced form, no changes were seen (not shown).

Figure 6 shows difference spectra of control and heated enzyme obtained upon partial reduction or oxidation. The spectrum of the heated enzyme (trace C) in the Soret region is clearly different from that of the intact enzyme (trace B). Beef heart cytochrome oxidase is known to show a spectroscopic (and probably conformational) change from the resting enzyme to a "pulsed state" upon reduction followed by oxygen addition (Antonini et al., 1977; Petersen & Cox, 1980). A similar spectroscopic change occurs more slowly and continuously during the aerobic steady state (Nicholls & Hildebrandt, 1978). In the case of the intact enzyme, immediately after the addition of ascorbate plus TMPD, a 10% reduction level is seen (trace D); this changes spectroscopically to a "pulsed" form with a Soret peak at 429 nm in the difference spectrum (trace B). The same pulsed spectrum is produced

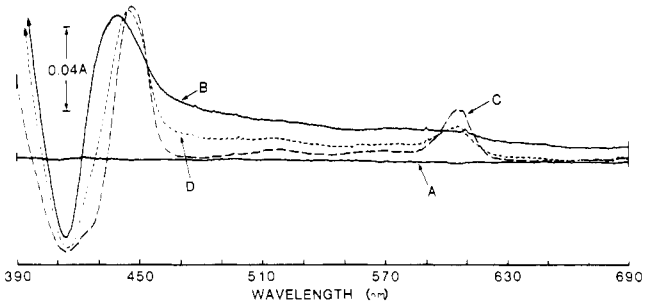


FIGURE 6: Effect of heat treatment on the spectral changes in cytochrome $aa_3$ upon pulsing the reduced form with oxygen: difference spectra. The resting enzyme was prepared as described in Figure 5, and its spectrum was used as a reference (A); ascorbate (10 mM) and TMPD (75 $\mu$M) were then added. After 10 min, anaerobiosis occurred, and then air was introduced by vigorous stirring (pulsed). The resulting spectrum was taken 30 s after the stirring. (A) Oxidized form (base line); (B) control without heat treatment; (C) heat-treated enzyme (43 °C, 40 min at pH 7.0); (D) control, not pulsed, spectrum taken immediately after the addition of ascorbate and TMPD under aerobic conditions.

immediately by pulsing the fully reduced enzyme with oxygen. But with heated enzyme, only a small shift (445 → 443 nm) was seen during the steady state or upon pulsing (trace C). The initial steady-state partially reduced spectrum of the heated enzyme was identical with that of the intact enzyme (not shown). Further work is clearly necessary to determine the significance of this change.

*Effect of Heat Treatment on $E_m$ Value of Cytochrome a.* The subunit III deficient enzyme shows neither a $H^+$-pumping capability nor a pH-dependent $E_m$ for cytochrome *a* in the presence of cyanide (Saraste et al., 1981). Penttilä (1983) reported that the apparent $E_m$ of cytochrome *a*, measured with a $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ couple in the presence of 3 $\mu$M cytochrome *c* and 2 $\mu$M cyanide, declines from +340 (pH 6.5) to +300 mV (pH 8.0), while that of subunit III deficient enzyme remains at +310 mV throughout this pH range. Figure 7 shows the result of a similar experiment with intact and with heat-treated enzyme. Both forms show a decline of between 10 and 20 mV over a pH range of 1.5 units; the difference between the two does not appear to be significant (see Discussion).

## Discussion

The data presented here show that there is a "magic" temperature zone in which beef heart cytochrome oxidase loses its $H^+$-pumping capability without affecting its transmembrane electron-transfer activity. We have previously reported a similar selective heat denaturation obtained by incubating bacterial cytochrome oxidase from the thermophile PS3 at 55 to ~60 °C (Ogura et al., 1984). The fact that $H^+$ pumping by cytochrome oxidase is more labile than oxidase activity may account for earlier observations in which no $H^+$ pumping (or only a trace of $H^+$ ejection) was observed, although the same reconstituted proteoliposomes exhibited a good respiratory control ratio. The present data indicate that liposomes containing the heated enzyme also retain their respiratory control and form a membrane potential almost as large as that produced by vesicles containing the intact enzyme (Figure 4).

The recent suggestion that cytochrome oxidase may not be a true proton pump postulates that $H^+$ ejection from cytochrome *c* oxidase containing liposomes following a reduced cytochrome *c* pulse is due either to a scalar deprotonation upon oxidation of cytochrome *c*–phospholipid complex (Mitchell & Moyle, 1983) or to a redox-linked rupture of a salt bridge releasing $H^+$ (Papa et al., 1983). But the present results clearly

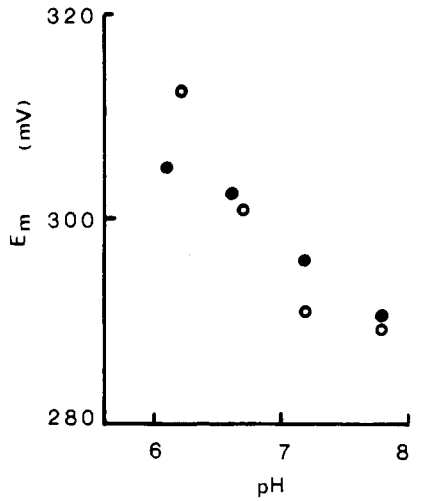


FIGURE 7: pH dependence of the apparent midpoint potential of cytochrome *a* in intact and heat-treated enzyme. The enzyme preparations were incubated in 20 mM K-MOPS (pH 6.9) containing 10 mM $K_2SO_4$, 0.5 mM KCN, and 0.25% Tween 80 at a final concentration of 1.8 $\mu$M cytochrome $aa_3$ for 20 h at 4 °C. Horse heart cytochrome *c* was added to give a final concentration of 2.6 $\mu$M. After the pH was adjusted to give the indicated value by MOPS or Tris, a 25-$\mu$L aliquot of potassium ferrocyanide/ferricyanide mixture (80 mM + 2 mM) was added to the cuvette containing 2.5 mL of the enzyme solution. The spectrum (650 nm – 500 nm) was recorded with the cyanide-inhibited fully oxidized form as base line. Then, small aliquots at 10–400 mM ferrocyanide were added to give several points from 324 to 264 mV (calculated from the $E_m$ value of 420 mV for the ferricyanide/ferrocyanide couple), and the spectrum was recorded each time. Finally, the fully reduced spectrum was obtained by the addition of $Na_2S_2O_4$. The $E_m$ was obtained graphically from the $E_h$, which gave half-reduction of cytochrome *a* (604 nm – 630 nm) or cytochrome *c* (550 nm – 540 nm). Cytochrome *c* showed a pH-independent $E_m$ value of 245 ± 5 mV. (○) Intact enzyme; (●) heat-treated enzyme.

indicate that the observed $H^+$ ejection cannot be a simple consequence of cytochrome *c* oxidation, since the latter also occurs after the mild heat treatment. Moreover, the method normally used to measure the $H^+/e^-$ ratio in our work is an initial rate technique; a small amount of cytochrome *c* is added as an electron mediator from ferrocyanide; and during the reaction, the cytochrome *c* turns over several times and the oxidase at least 10 times. These experiments support previous observations (Wikström & Saari, 1977; Sigel & Carafoli, 1978; Casey et al., 1979; Sone & Hinkle, 1982; Proteau et al., 1983) that indicate that the apparent $H^+$ ejection is due to an authentic $H^+$ pumping by cytochrome *c* oxidase.

At least two other methods have been reported to inhibit $H^+$-pumping capability without appreciably affecting oxidase activity. Removal of subunit III of the beef heart enzyme (Saraste et al., 1981; Penttilä, 1983) and *N,N'*-dicyclohexylcarbodiimide treatment (also modifying subunit III; Casey et al., 1980; Prochaska et al., 1981) both induce a loss of $H^+$ ejection. Each of these methods affects subunit III, which is assumed to be the $H^+$ channel portion of cytochrome *c* oxidase (Wikström & Saari, 1977; Wikström & Krab, 1979) rather like $F_0$ (the membrane sector) in $H^+$-ATP synthetase ($F_0F_1$). In contrast, the present simple method may inactivate the $H^+$-pumping capability in a different way, by modifying the $H^+$-pumping machinery at a point closer to the site of oxidation–$H^+$ translocation coupling. The observation that the $E_m$ change of cytochrome *a* with pH is unaffected by heat treatment (Figure 7), while cytochrome *a* in subunit III deficient enzyme shows only little, if any, pH-dependent $E_m$ change (Penttilä, 1983), supports the idea that the point inactivated by heat could be different from subunit III. How-

ever, the pH dependence of $E_m$ is rather small (both in Penttilä's work and in our work), and such a conclusion is therefore necessarily very tentative.

A spectral change was also observed in the Soret region of the oxidized (resting) form of the enzyme upon heat treatment, although no change occurred in the $\alpha$ region (Figure 5). Resonance Raman studies of PS3 cytochrome oxidase have shown that the reduced form of the enzyme incubated at 60 °C, which retains oxidase activity but not $H^+$-pumping capability, also loses the Raman line at 213 $cm^{-1}$ (Ogura et al., 1984). This Raman line has been assigned to Fe($a_3$)–N-(histidine) stretching (Ogura et al., 1983). A similar change has been observed in beef heart enzyme incubated at 43 °C (T. Ogura and N. Sone, unpublished observations), although further experiments are required to correlate the Raman changes with other changes in enzymatic properties. It may be noted that the heat-treated beef heart enzyme does not show a typical spectral change upon pulsing (Figure 6). Thus, some change seems to have taken place in the vicinity of cytochrome $a_3$ heme, which could be the cause of the uncoupling of $H^+$ pumping. On the other hand, we have not ruled out the possibility that all these effects are the indirect results of a functional dissociation of a subunit III. Since heat treatment at 43 °C may not be specific, a further investigation, and a more quantitative one, may be necessary. However, this selective heat-treatment technique does seem useful, not only to establish the $H^+$-pump capability of cytochrome *c* oxidase but also to give us clues as to how cytochrome oxidase actually works as a proton pump.

## Acknowledgments

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**Registry No.** Hydrogen ion, 12408-02-5; cytochrome *c* oxidase, 9001-16-5.

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