Enzymatic Properties of Cytochrome Oxidase from Bovine Heart and Rat Tissues

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Abstract: Cytochrome oxidase was purified from bovine-heart mitochondria and its enzymatic properties were examined. The purified cytochrome oxidase was identified by its absorption spectrum and chromatogram through gel filtration. The specific activity, purification degree and yield of purified cytochrome oxidase were 18 nmol/mg/ml/min, 24.83 fold and 0.93%, respectively. The activity of the enzyme assayed by a ferrocytochrome c-O_2 system was optimized at 25℃ and pH 6.5. Examining the effect of nonionic detergents established that cytochrome oxidase was deactivated by Triton X-100. The oxidase was activated by Tween 80 and deactivated by Tween 20. The Michaelis constant and maximum velocity of the oxidase for ferrocytochrome c were 0.032~0.044 mM and 0.019~0.021 mM/min, respectively. After adaption to basal diet for a week, experimental diets containing 6 mg Cu/kg, or zero mg Cu/kg, or 12 mg Cu/kg were fed to a control group, a copper-free group and a copper-rich group of Sprague-Dawley rats, respectively, for 4 weeks. The specific activities assayed for the ferrocytochrome c-O_2 system of isolated cytochrome oxidase from the rat liver of control, copper-free, and copper-rich group were 1.00, 1.19, and 0.878 nmol/mg/ml/min, respectively. Their degrees of purification were 11.38, 10.82 and 8.78 fold, respectively. The specific activities for liver and heart mitochondrial cytochrome oxidase of copper-free/copper-rich groups assayed using the ferrocytochrome c-O_2 system were 81.4% and 96.4%/64.1% and 61.1%, respectively, compared with those of the control.

Key words: copper diet, cytochrome oxidase, electron transport, ferrocytochrome, mitochondria.

The electrons in NADH and $FADH_2$ are furnished to oxygen through the actions of some electron-transport complexes in mitochondrial inner membrane for use in ATP production. The electron transport via the inner membrane complexes alone has been designated the internal electron transport of mitochondria (Mitchell, 1979; Malmstrom, 1989). Bernardi and Azzone (1981) proposed the existence of external electron transport through mitochondrial outer membrane containing an enzyme complex of NADH-cytochrome b_5 reductase which would transfer an electron of external NADH to cytochrome b_5. They also described how cytochrome b_5 donated its electron via an cytochrome c electron shuttle present in intermembrane space between mitochondrial inner and outer membranes. In studies of bioenergy transduction through a mitochondrial electron transport system like this, individual research on the components making up the electron transport system is required. Of the many components in the electron transport system, cytochrome oxidase especially acts as the finishing transport site catalyzing the electron transport from cytochrome c to oxygen coupling with proton translocation. The properties of bovine-heart cytochrome oxidase isolated using detergent were examined in terms of its reactivity under various conditions. This enzyme complex contains copper, which has been considered to regulate the electron transport rate of the enzyme but to be not essential for oxygen reduction (Nilson *et al.*, 1988). Lawrence *et al.* (1985) also supposed that the reactivity of the enzyme may be controlled by the metal. Also, experimental diets of different copper contents were fed to rats to compare the activity of cytochrome oxidase from liver and heart tissues.

Materials and Methods

Materials

Fresh bovine heart was obtained from a local slaughterhouse for the separation of mitochondria. Male adult Sprague-Dawley rats (200~250 g) were provided from the breeding room in the Medical School of Yeungnam University. Hearts and livers for the separation of mito-

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chondrial cytochrome oxidase were taken from these animals after the administration of the experimental diets for four weeks.

Sodium phosphate monobasic and dibasic, sucrose, potassium chloride, ammonium sulfate, sodium dithionite, Tween 20, Tween 80 were obtained from Junsei. Tris (hydroxymethyl) aminomethane, sodium deoxycholate, cytochrome c (horse heart, typeIII, sodium salt) were purchased from Sigma Chemical Co. L-histidine was purchased from Hayashi, and albumin (bovine) was a product of Fluka.

Mitochondria separation

A method for the purification of the mitochondrial inner-membrane enzyme complex was used (Ragan *et al.*, 1987) to separate the mitochondria from bovine heart.

Rat mitochondria were prepared by the method of Kwak and Kwak (1986) as follows. The animals fed on the experimental diet were slightly anesthetized with ether and stunned by physical shock in order to cut tissues out. The heart and liver were finely minced to make separate homogenates adding severally 0.25 M sucrose to a volume 9-fold that of each sliced tissue. These several homogenates were centrifuged at $571\times g$ for 10 min to obtain the supernatant and the pellets, which were clots containing nuclei, were discarded. The supernatants from the above were recentrifuged at $7{,}796\times g$ for 20 min to obtain mitochondria-containing pellets for the fractionation described below. The pellets were suspended in small volumes of 0.25 M sucrose to load them upon two-layer solutions formed by sucrose density of 20 and 45 W/V % and were then spun at $45{,}200\times g$ for 20 min. The sediments from the spinning were dispersed in 0.25 M sucrose and recentrifuged at $7{,}796\times g$ for 20 min for the purpose of obtaining mitochondrial fractions.

Three groups of rats were raised as described below. We drew two tissues, liver and heart, from each group.

Animal raising

Thirty rats described above were adapted to basal diet (composition not shown) for one week and then grouped in three groups of ten. The animals of the several groups were fed on experimental diets of different copper contents. The copper compound fed was $CuSO_4\cdot 5H_2O$. The Control group took a 6 mg Cu/kg diet following the standard of the American Institute of Nutrition, i.e., AIN-76™ (1977) for 4 weeks. The copper-rich group took a 12 mg Cu/kg diet for an identical period under the condition described below. The copper-free group was also raised under this condition.

All the equipments for the raising, including especially food containers, were washed with 0.4% EDTA repeatedly. Animals were raised under the temperature of (20±2) ℃ with alternate 12 h lighting and 12 h darkness per day. Water distilled twice was used for drinking water and much was supplied. Rats were starved for 16 h before being killed.

Purification of cytochrome oxidase from the mitochondria of bovine heart and rat liver

The enzyme purification was performed combining the methods of Fowler *et al.* (1962), Wharton and Tzagoloff (1967), and Errede *et al.* (1978) as follows. The mitochondrial pellet obtained by centrifugation was well dispersed in a small quantity of 0.25 M sucrose and centrifuged at $78{,}500\times g$ for 20 min. The pellet obtained was dispersed in a buffer solution of TSH (50 mM Tris, 0.67 M sucrose, 1 mM histidine, pH 8.0) to make a suspension of 23 mg protein/ml. To this TSH suspension was added 0.3 mg sodium deoxycholate/mg protein and 72 g potassium chloride/l. The suspension supplemented with the salts was spun at 78,500xg for 30 mins to obtain a green pellet. The pellet was dispersed in the same volume of TSH buffer solution to be respun at $78{,}500\times g$ for 30 min. After eliminating the supernatant, the middle portion of sediment was taken out carefully and dispersed it in TSH buffer solution to make a protein suspension of 19 mg/ml. To this suspension 0.5 mg sodium deoxycholate/mg protein and 74.5 g potassium chloride/l were added to make a suspension to be spun at $78{,}500\times g$ for 10 min. The supernatant from this spinning was dialyzed against 0.01 M Tris-HCl, pH 8.0 for 90 min. A volume of 0.19 ml of saturated ammonium sulfate solution per ml of dialyzate was added to the dialyzate and the mixture was stirred while kept cool by ice-cooled water for 10 mins and then centrifuged at $78{,}500\times g$. To the supernatant solution we added 0.04 ml of saturated ammonium sulfate solution per ml of the supernatant to cause precipitation. Another centrifugation at $78{,}500\times g$ for 10 min and another precipitation were carried out to obtain a pellet of enzyme protein source, which was stored below −60℃. The mitochondria separation and the cytochrome oxidase purification were conducted at 4℃ or below throughout the total procedure.

Identification of cytochrome oxidase

Purified cytochrome oxidase was dissolved in 0.1 M Tris-HCl, pH 8.0, containing 1% sodium deoxycholate to observe the electronic absorption spectrum for the oxidized form (Wrigglesworth *et al.*, 1988). To the enzyme cytochrome a minute amount of sodium dithionite

was added at 0℃ to observe its spectrum for the reduced form at the same time.

A gel-filtration chromatography using a Protein-Pak column (Waters) was performed for an enzyme-purity inspection. The mobile phase used for this filtration was a buffer solution of 0.05 M potassium phosphate, pH 6.0. Ten microliters of enzyme solution was loaded upon the column using a U6k Universal Injector (Waters) and eluates were monitored at 280 nm with a UV/VIS Absorbance Detector-M440 (Waters).

Activity assay for cytochrome oxidase

Cytochrome oxidase activity was assayed using the method described by Wharton and Tzagoloff (1967) as follows. The mixture composed of 100 μl of 0.1 M sodium phosphate buffer solution, pH 7.0, and 70 μl of 1.204×10^{-4} M ferrocytochrome c was diluted to 1 ml with water. The reaction was initiated with the addition of 10 μl of 0.33 mg/ml cytochrome oxidase solution. The rate was measured at 25℃ immediately in terms of the absorption decrease at 550 nm where the α-band of ferrocytochrome c exists. The measuring was carried out by the method of time driving or overlaying performed automatically by UV/VIS spectrophotometer. A blanc test for the redox reaction in the ferrocytochrome-oxygen system without the enzyme was also conducted. All the reaction rates measured were steady-state initial velocities.

Ferricytochrome c was dissolved in a solution of 0.01 M sodium phosphate, pH 7.0, and reduced by the addition of a small amount of sodium dithionite, a reductant. This protein solution was dialyzed against a buffer solution of 0.01 M sodium phosphate, pH 7.0 for 18 h before enzyme catalysis.

Dependence of the activity on pH, temperature, surfactants and substrate concentrations

The pH of the activity-assay system for the oxidase was made to be 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 with a buffer solution of 0.1 M sodium phosphate to measure enzyme-activity dependence on pH. The temperature dependency of the activity was also examined by checking the 550 nm-absorbance change at 20°, 25°, 30°, 40°, 45°, and 50℃. Nonionic surfactants Triton X-100, Tween 80, and Tween 20 were added severally to the activity-assay system in 1% concentration to examine the activity-changing effects exhibited by the surfactants. Substrate cytochrome c in concentrations of 0.02, 0.03, 0.04, and 0.05 mM was added to the enzyme-catalysis system to measure the several rates. This measurement gave the Michaelis constant and maximum velocity.

Protein quantification and significance test

The biuret method introduced by Rendina (1971) and Layne (1957) was used for quantifying protein employing a calibration curve drawn with bovine serum albumin as a standard. The significance test employed was Duncan's multiple range test.

Results and Discussion

The reports on cytochrome oxidase separation from mammalian eukaryotic cells frequently described ammonium-sulfate precipitation after membrane breakup with detergents (Griffiths, 1961; Takemori *et al.*, 1962; Yonetani, 1961; Fowler *et al.*, 1962; Horie *et al.*, 1963; Wainio, 1964). Other workers introduced adsorption chromatography utilizing a specific interaction between cytochrome oxidase and electron-donor cytochrome c, and ion-exchange chromatography (Rascati and Parsons, 1979; Broger *et al.*, 1986). We used the method combining sodium deoxycholate treatment, protein precipitation with ammonium sulfate and centrifugation for the purpose of cytochrome oxidase isolation (Table 1). The second ammonium sulfate precipitation as the last purification step gave us cytochrome oxidase whose specific activity was 18 nmol/mg/ml/min. This corresponded to a 24.83-fold purification and 0.93% yield.

Also, we ascertained a considerable enzyme activity without adding any phospholipid to the enzyme-assay system. This suggests the possibility that our so-called

Table 1. Purification profile for cytochrome oxidase from bovine heart mitochondria.

Step	Protein (mg)	Specific activity[a] (nmol/mg/ml/min)	Total activity	Purification (fold)	Yield (%)
Mitochondria	1288	0.725	933.8	1	100
1st treatment of deoxycholate and KCl	618	1.70	1050.6	2.34	48
2nd treatment of deoxycholate and KCl	242.12	0.841	203.62	1.16	18.8
Dialysis	274.5	1.97	540.77	2.72	21.31
1st treatment of ammonium sulfate	113.75	1.889	214.87	2.61	8.83
2nd treatment of ammonium sulfate	12	18	216	24.83	0.93

[a]The specific activity is defined as amounts of the oxidized cytochrome c per mg of protein reacted for 1 min at 25℃.

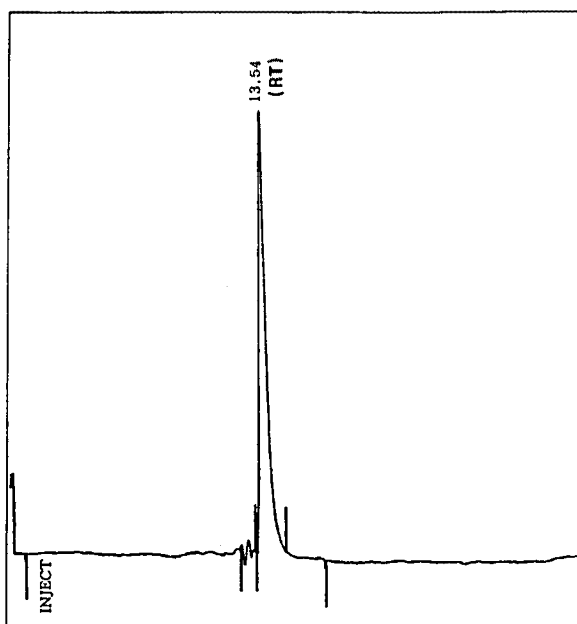

Fig. 1. Elution profile of gel-filtration HPLC for the purified cytochrome oxidase from bovine heart mitochondria. The column used is a protein pak columm I-125 and developed with 0.05 M potassium phosphate, pH 6.

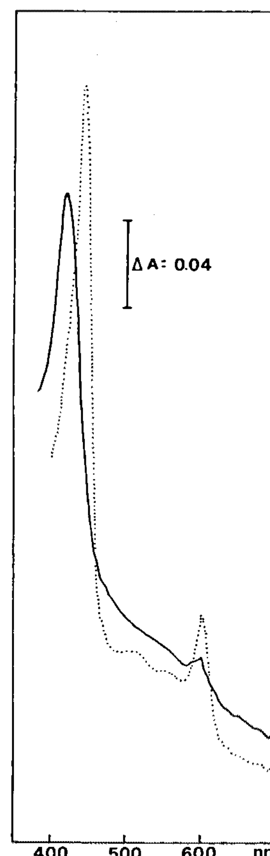

Fig. 2. Absolute absorption spectra of reduced (dot line) and oxidized (solid line) cytochrome oxidase preparation. Purified cytochrome oxidase was suspended in 0.1 M Tris-HCl (pH 8.0) containing 1% sodium deoxycholate and reduction was done by sodium dithionite under the same medium at 0℃.

purified enzyme could be a phospholipid-containing oxidase. The eluted purified oxidase showed a single band with no other protein contamination through the gel-filtration column of Protein-Pak with a retention time of 13 min (Fig. 1).

The absorption spectra of this enzyme were examined at length for the sake of enzyme identification, the differentiation between cytochrome a and a_3, reaction-specificity comparison for the subunits, concentration determination based on heme, a component (Griffiths and Wharton, 1961; Yonetani, 1961; Bill *et al.*, 1980; Vrij *et al.*, 1982; Sone, 1986). Oxidized cytochrome oxidase from our purification gave absorption bands at 598 nm and 421 nm. The reduced oxidase in this study gave bands at 604 nm and 443 nm which are the α and γ bands respectively characteristic for heme a (Fig. 2). These spectral profiles are identical with those in the other studies mentioned above. The spectral outlines of the enzyme preparation from our purification were also identical with those from other sources mentioned below in accordance with the information. The reference spectra we compared were those of the oxidases from the sources of shark (Wilson *et al.*, 1980), Baker's yeast (Mason,1973), and human heart (Sinjorgo *et al.*, 1987). The assay of the oxidase was conducted by measuring oxygen consumption or cytochrome oxidation (Yonetani, 1962). Our assay was carried out by checking the 550 nm-absorption decrease for ferrocytochrome c in the presence of oxygen. The blanc test for the ferrocytochrome c-O_2 system gave confidence that the cytochrome could not be oxidized without the enzyme, whereas the substrate in the system could be oxidized by the addition of the enzyme (Fig. 3). The activity of cytochrome oxidase in various buffer solutions of 0.01 M sodium phosphate of varying pH was examined in the ferrocytochrome c-O_2 system. It was observed that the enzyme was most active at pH 6 to pH 6.5 (Table 2). Temperature dependence of the activity was monitored equilibrating the reaction system at several temperatures followed by adding the enzyme. The specific activity at 25℃ was larger than that at any other temperature examined (Table 3). Wainio *et al.* (1960) examined the cytochrome oxidase activity in KH_2PO_4-Na_2HPO_4 buffer solution whose concentrations were 0.010, 0.025, 0.050, 0.075, 0.100, 0.150 and 0.200 M. They also checked the dependence of the activity on pH at these concentrations of the buffer solution. They observed an optimum pH of 6.0 which was irrespective of the concentrations of buffer solution, whereas enzyme activity somewhat changed depending on buffer-solution concentrations.

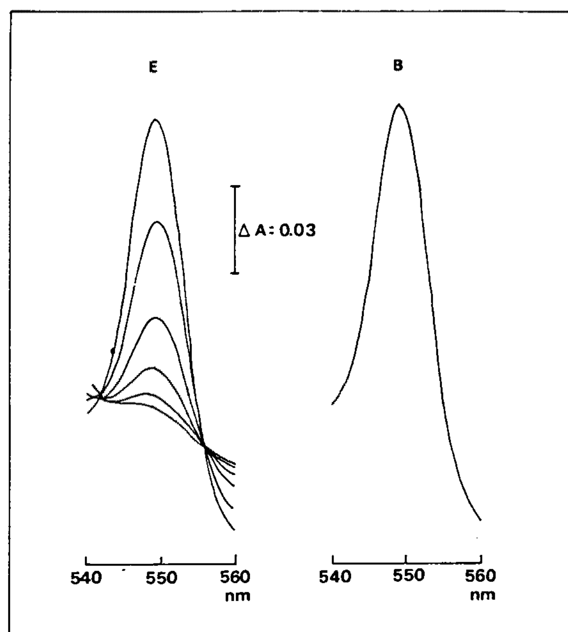

Fig. 3. The activity of cytochrome oxidase was measured as absorbance decrease in the range of 560~540 nm by overlaying or time driving of UV-VIS spectrophotometer. The overlaying was conducted six times with an interval of 2 min. E and B represent ferrocytochrome c-O_2 reaction system in the presence or absence of cytochrome oxidase, respectively.

The detergents added frequently to the reaction system of cytochrome oxidase were nonionic. Tween 80 was used more frequently than other nonionic detergents such as Triton X-100 and Tween 20. The effects of 1% Tween 80, 1% Triton X-100 and 1% Tween 20 on the activity of the oxidase were compared in this experiment. Triton X-100, when added to the reaction system lowered specific activity compared with the system lacking detergent, whereas the specific activity of the system of Tween 20 or Tween 80 was similar with or greater than that without a detergent (Table 4). Rascati *et al.* (1979) reported that the addition of 0.5% Tween 80 or the addition of 0.5% Tween 80 plus phospholipid to the reaction system of cytochrome oxidase from rat-liver mitochondria activated the enzyme, whereas the addition of 1% Triton X-100 deactivated the enzyme by 46%, compared with the control system. They also found that the effect of detergents or phospholipid on cytochrome oxidase activity depended on the substrates used and the species source of the enzyme. Yamazaki *et al.* (1988) examined the effect of anionic detergents and nonionic detergents on the reaction in a system composed of the cytochrome oxidase from *Nitrosomonas europaea* and ferrocytochrome c from horse, *Condida krusel*, *N. europaea*. The cytochrome oxidase reactivity using the electron donor of eukaryotic cytochrome c was elevated by the anionic detergents of sodium dodecyl sulfate and sodium cholate, while the reactivity was somewhat decreased by the nonionic detergents of Tween 20 and Triton X-100. They reported that the oxidation rate for *N. europaea* ferrocytochrome c-552 by the enzyme showed no considerable effect from the addition of any of the detergents described above. Though the mechanism for the evident reactivity change evoked by detergents was not clarified, researchers are supposing that these agents could change the aggregation state of the enzyme itself.

The Michaelis constant and maximum velocity, representing the affinity of the enzyme for substrate, were calculated through measuring the reaction rates at several ferrocytochrome c concentrations as follows (Fig. 4). Three methods modifying the Michaelis-Menten equation, that is, the Lineweaver-Burk reciprocal plot, the Hanes-Woolf plot and the Woolf-Augustinsson-Hofstee plot were employed to fix K_m and V_{max}. A least-square method was also introduced for statistical interpretation (Segel, 1976). The K_m of cytochrome oxidase

Table 2. The effect of pH on the cytochrome c oxidase activity in the ferrocytochrome c-O_2 reaction system.

pH	5.5	6.0	6.5	7.0	7.5	8.0
Specific activity[a] (nmol/min/ml)	5.68	10	12	7	6.8	5.84

[a]The specific activity is defined as amounts of the oxldized cytochrome c per ml of reaction mixture reacted for 1 min at 25℃ in the presence of 0.6 mg/ml cytochrome c oxidase.

Table 3. The effect of temperature on the cytochrome c oxidase activity in the ferrocytochrome c-O_2 reaction system.

Temperature (℃)	50	45	40	30	25	20
Specific activity[a] (nmol/min/ml)	0.308	0.249	0.974	1.36	3.11	2.21

[a]The specific activity is defined as amounts of the oxldized cytochrome c per ml of reaction mixture reacted for 1 min at each temperature in the presence of 0.6 mg/ml cytochrome c oxidase.

Table 4. The effect of nonionic detergents on the cytochrome c oxidase activity in the ferrocytochrome c-O_2 reaction system.

Detergent	Specific activity[a] (nmol/min/ml)
Control	3.35
1% Triton X-100	0.584
1% Tween 20	2.92
1% Tween 80	3.89

[a]The specific activity is defined as amounts of the oxidized cytochrome c per ml of reaction mixture reacted for 1 min at 25℃ in the presence of 0.6 mg/ml cytochrome c oxidase.

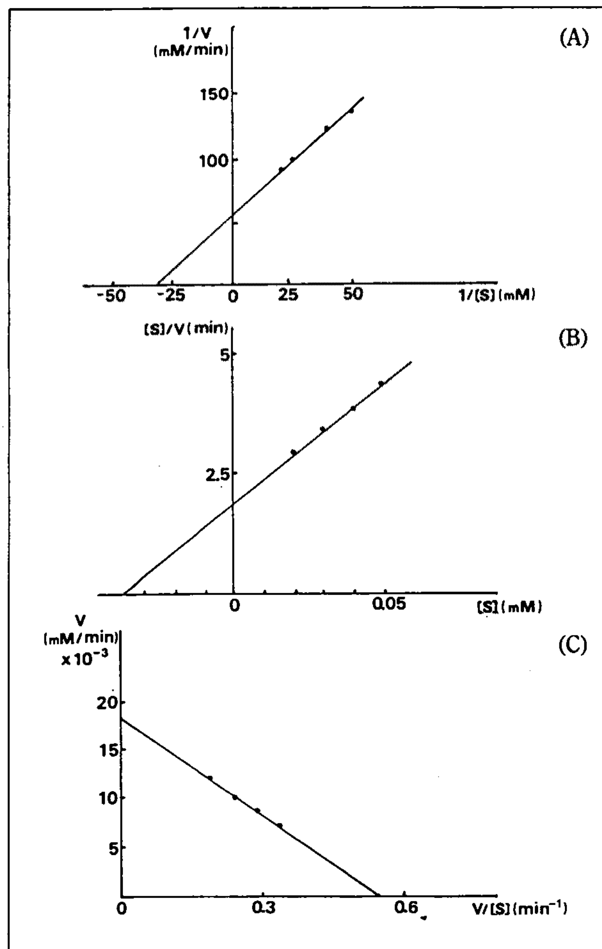

Fig. 4. Lineweaver-Burk reciprocal plot (A) and Hanes-Woolf plot (B) and Woolf-Augustinsson-Hofstee plot (C) for the determination of the parameters K_m and V_{max} of purified cytochrome oxidase. These reactivities of the oxidase were estimated in the ferrocytochrome c-O_2 reaction system. From the above plots, K_m and V_{max} were confirmed to be 0.032~0.044 mM and 0.019~0.021 nmol/min, respectively. In this calculation, least square determination method was employed.

from bovine-heart mitochondria for ferrocytochrome c and its V_{max} were 0.032~0.044 mM and 0.019~0.021 nmol/min, respectively.

Cohen and Elvehjem (1934) reported the implication of copper in cytochrome oxidase activity in their nutritional study on copper. They found that the hepatic cytochrome oxidase activity of rats fed with copper-free milk was decreased. Lawrence *et al.* (1985) raised two groups of rats which were fed on a 5 mg Cu/kg diet and 0.5 mg Cu/kg diet for 2, 4, 6 and 8 weeks. They called the former the copper-adequate control group and the latter the copper-deficient group. They examined the copper and iron concentrations in plasma and liver from each group. They also examined the concentrations of copper, iron, cytochrome protein; cytochrome oxidase activity and fatty-acid composition within hepatic mitochondria from each group. Our experiment raised three groups of rats. The normal control group took a 6 mg/kg diet of copper after AIN-76™ (1977). The group called the CE group were fed a copper-free diet and the group called the CL group took a diet containing 12 mg Cu/kg diet. Diets were continued for four weeks under equivalent conditions for the control, CE and CL groups to measure the reactivity of cytochrome oxidase from liver and heart intact mitochondria (Table 5), and to measure the activity of the enzyme purified from liver. The specific activities of the purified hepatic cytochrome oxidase from the control group, CE group, and CL group were 1.00, 1.19 and 0.878 nmol/mg/ml/min, respectively. These correspond to 11.38, 10.82, and 8.78-fold purification, respectively Table 6). The body weights of rats used in this experiment were from 200 to 250 g, which were thought to be those of fully grown animals. It is supposed that these grownup rats were little affected by oral copper deficiency. Younger rats weighing under 100 g with active metabolism could be seriously affected by the diet level of copper. On the contrary copper administration to the animals resulted in a substantial activity decrease of the heart oxidase for the rats which were fed a the diet containing twice as much of the metal as in the control diet, in contrast with the minor oxidase-activity decrease for the animals fed a deficient copper diet.

Table 5. The activity of rat liver/heart mitochondrial cytochrome c oxidase in the ferrocytochrome c-O_2 redox system.

	Control	CE	CL
Specific activity[a] (mol/mg/min)	3.06/2.47	2.49/2.38	1.96/1.51
Relative activity (%)	100/100	81.4/96.4	64.1/61.1

[a]The specific activity is defined as amounts of the oxidized cytochrome c per mg protein concentration per ml of reaction mixture reacted for 1 min at 25℃.

Table 6. The comparison of purified cytochrome c oxidase activities from rat liver of the control, CE and CL group.

	Specific activity[a] (nmol/mg/ml/min)	Purification (fold)
Control	1.00	11.38
CE	1.19	10.82
CL	0.878	8.78

[a]The specific activity is defined as amounts of the oxidized cytochrome c per ml of protein reacted for 1 min at 25℃.

References

American Institute of Nutrition (1977) *J. Nutr.* **107**, 1340.

Bernardi, P. and Azzone, G. F. (1981) *J. Biol. Chem.* **256**, 7187.

Bill, K., Casey, R, P., Broger, C. and Azzi, A. (1980) *FEBS Letters* **120**, 248.

Broger, C., Bill, K. and Azzi, A. (1986) *Methods Enzymol.* **126**, 64.

Cohen, E. and Elvehjem, C. B. (1934) *J. Biol. Chem.* **107**, 97.

Errede, B., Kamen, M. D. and Hatefi, Y. (1978) *Methods Enzymol.* **53**, 40.

Fowler, L. R., Richardson, S. H. and Hatefi, Y. (1962) *Biochim. Biophys. Acta.* **64**, 170.

Griffiths, D. E. and Wharton, D. C. (1961) *J. Biol. Chem.* **236**, 1850.

Horie, S. and Morrison, M. (1963) *J. Biol. Chem.* **238**, 1855.

Kwak, C. S. and Kwak, J. S. (1986) *Thesis Collection of Kyemyoung Med. School* **5**, 45.

Lawrence, C. B., Davies, N. T. and Nicol, F. (1985) *Biochim. Biophys. Acta.* **809**, 351.

Layne, E. (1957) *Methods Enzymol.* **3**, 449.

Malmstrom, B. G. (1989) *FEBS Letters* **250**, 9.

Mason, T. L. (1973) *J. Biol. Chem.* **248**, 1346.

Mitchell, P. (1979) *Science.* **206**, 1148.

Nilson, T., Copeland, R. A., Smith, P. A. and Chan, S. I. (1988) *Biochemistry* **27**, 8254.

Ragan, C. I., Wilson, M. T., Darley-Usmar, V. M. and Lowe, P. N. (1987) in *Mitochondria*, pp. 79-112, IRL press, Oxford.

Rascati, R. and Parsons, P. (1979) *J. Biol. Chem.* **254**, 1586.

Rendina, G. (1971) in *Experimental Methods in Morden Biochemistry*, pp. 75-77, W. B. Saunders company, Philadelphia.

Segel, I. H. (1976) in *Biochemical Calculations*, pp. 208-323, John Wiley and Sons, Inc, Toronto.

Sinjorgo, K. M. C., Hakvoort, T. B. M., Durak, I., Draijer, J. W., Post, J. K. P. and MuijSers, A. O. (1987) *Biochim. Biophys. Acta.* **850**, 140.

Sone, N. (1986) *Methods Enzymol.* **126**, 145.

Takemori, S., Sekuzu, I. and Okunuki, K. (1961) *Biochim. Biophys. Acta.* **51**, 464.

Vrij, W. D., Azzi, A. and Konings, W. N. (1982) *Eur. J. Biochem.* **125**, 189.

Wainio, W. W., Eichel, B. and Gould, A. (1960) *J. Biol. Chem.* **235**, 1521.

Wainio, W. W. (1964) *J. Biol. Chem.* **239**, 1402.

Wharton, D. C. and Tzagoloff, A. (1967) *Methods Enzymol.* **10**, 245.

Wilson, M. T., Lalla-Maharajh, W., Darley-Usmar, V., Bonaventura, J., Bonaventura, C. and Brunori, M. (1980) *J. Biol. Chem.* **255**, 2722.

Wrigglesworth, J. M., Elsden, J., Chapman, A., van der Water, N. and Grahn, M. F. (1988) *Biochim. Biophys. Acta* **936**, 453.

Yamazaki, T., Fukumori, Y. and Yamanaka, T. (1988) *J. Biochem.* **103**, 499.

Yonetani, T. (1961) *J. Biol. Chem.* **236**, 1680.

Yonetani, T. (1962) *J. Biol. Chem.* **237**, 550.