Cytochrome Oxidase Activity of Individual Mitochondrion as Quantified by Platinum-Diaminobenzidine Reaction with Energy Dispersive X-ray Analyzer

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The platinum diaminobenzidine (Pt-DAB) reaction was characterized by the oxidative polymerization of DAB with a concurrent incorporation of Pt atoms into the polymerization product in the presence of cytochrome oxidase activity. It conformed to the Michaelis-Menten's formula showing its enzymatic nature. It was specific to the oxidase as proved by the suppressive effects due to some inhibitors and heat. It permitted the quantification of the oxidase activity of an individual mitochondrion and its constituent part by energy dispersive X-ray analysis (EDAX) owing to the incorporated Pt atoms. To elucidate the gradient in the oxidase activity along the radial axis of a liver lobule, the local activity difference between the hepatocytes in the peripheral region (PE) adjacent to the triad and those in the central region (CN) close to the central vein was quantified at individual mitochondrial level. The mitochondria in the PE hepatocytes were found 1.5 times stronger in activity per area of the reactive site than those in the CN hepatocytes.

Key words: Cytochrome oxidase, Pt-DAB reaction, EDAX, Local activity difference in liver lobule

I. Introduction

An improved platinum-diaminobenzidine (Pt-DAB) reaction for quantitation of cytochrome oxidase activity has been developed. It yields a black, practically insoluble, electron dense, Pt-containing reaction product directly at the active site of the oxidase without postosmification [7]. Based on the Pt atoms incorporated in the product, it permits us to quantify the oxidase activity by energy dispersive X-ray analysis (EDAX) [2, 16 and 17]. This paper deals with its cytochemical features and its use in quantitation of the activity at individual mitochondrial level in hepatocytes of different location, peripheral and central, in the liver lobule.

II. Material and Methods

Material

Adult mice of specific pathogen free MCH (ICR) strain were used. After the animal was anesthetized with Nembutal, the liver was perfused from the portal vein to the inferior vena cava with the physiological saline solution, then with 2.5% glutaraldehyde dissolved in 0.1 M phosphate buffer solution of pH 7.4 for 5 min. It was excised, cut into small blocks, immersed in the aldehyde solution for another 5 min, and finally washed overnight with the buffer at 4°C under shaking. Frozen sections were prepared from these blocks with a freezing microtome. Those sections, 15 μm thick, were used as the specimens to check cytochemical characteristics of the reaction, while those, 10-80 μm thick, to test the reagent penetration into them by microspectrophotometry. The former sections were also used as the specimens for the solubility test of the reaction product by extraction. Heat-treated sections, 15 μm thick, which had been kept at 80°C for 15 min, were used to prove the reaction specificity. Besides these frozen sections, non-frozen liver slices were cut from the aldehyde-fixed blocks with a microslicer (Dōsaka E.M., Kyoto) at 40 μm, and they served as the specimens to...
prepare semithin sections for microspectrophotometric studies and ultrathin sections for morphometric and EDAX ones.

Reagents

The Pt-DAB reagent for cytochrome oxidase consisted of 0.2 M sucrose, 0.25 mg/ml cytochrome c (Type III, Sigma Chem. Co., St. Louis), 0.1 mg/ml catalase (Sigma), 2.5 mM 3,3'-diaminobenzidine tetrahydrochloride (DAB, Dötite, Dōjin Chem. Lab., Kumamoto), and 1 × 10^{-2} M platinous potassium chloride (PtK₂Cl₄, Nakalai Tesque, Kyoto) dissolved in 0.1 M phosphate buffer of pH 7.4 (cf. 19). Filtration through filter paper was unnecessary, if PtK₂Cl₄ was dissolved lastly. This reagent was temperature sensitive, and should be prepared under cooling just prior to the reaction. It was stable for 5 hr at 4°C, for 30 min at 25°C and for 15 min at 37°C. Modified Pt-DAB reagents of different Pt to DAB ratios, those of various DAB, cytochrome and catalase concentrations, those supplemented with KCN and with NaN₃, and those of different pH values were prepared for specified purposes. Here, the pH values were adjusted by McIlvaine's buffer [3]. Three kinds of blank reagents, the reagent deprived of Pt (DAB reagent), that deprived of DAB (Pt medium) and that deprived of both Pt and DAB (basal medium) were also prepared.

Reaction procedure and microspectrophotometry

Liver sections, including both frozen and non-frozen ones, were treated with the Pt-DAB reagent or with each of the blank ones for 45 min at 25°C under gentle shaking with reagent renewal at every 15 min interval. Besides this standard procedure, the reaction was carried out for different durations, at different temperatures and with modified reagents to check the optimal reaction parameter. All the stained and the control sections were dehydrated by ethanol, immersed in xylol and mounted in Biolite (Oken, Tokyo). A scanning microspectrophotometer equipped with an integration unit (MMPS-TU, Olympus Opt. Ind. Co., Tokyo) was used to study the absorption spectrum of the reaction product and to estimate the reaction intensity. The integrated absorbance was obtained by scanning an assigned field of cytoplasm with a monochromatic light spot of 2 μm diameter for 6 μm distance. This field covered the cytoplasmic area of 15.14 μm². The measurement was made at the wave length of 500 nm against the control section treated with the basal medium [8].

Solubility of reaction product

The stained and the control sections of known area (a, ca. 100 mm²) were blotted, homogenized with 500 μl volume of the below-mentioned solvents at 20°C. The homogenate was spun with a microcentrifuge at 5,000 g for 5 min, and the absorbance of the resulting supernatant from the former (A_{sup}) was determined against the blank one from the latter with a microcolorimeter after Malström & Glick [13]. Capillary cuvettes of 70 μl volume with 1 cm path were used. Concurrently, the permanent preparations of the corresponding stained and the control sections were prepared, and the absorbance of the former (A_{sec}) was determined microspectrophotometrically against the latter. The solubility of the reaction product in a given solvent was expressed by the ratio:

S = (A_{sup} \cdot (500/70)/(A_{sec} \cdot a \cdot e)) \times 100.

Here, e was an areal ratio of the cytoplasm positive in the oxidase activity to the whole section, as estimated by morphometry mentioned below. The solvents tested were ethanol (100%), acetone, 1:1 mixture of acetone and nonenyl succinic anhydride (NSA, Polyscience Inc.), methylethylketone (MEK), N,N'-dimethylformamide (DMFA) and dimethylsulfoxide (DMSO).

Semithin sections for microspectrophotometry

Non-frozen liver slices, 40 μm thick, were subjected to the Pt-DAB reaction after the standard procedure. Both the stained and the control slices were washed for 30 min at 4°C with the buffer under gentle shaking, dehydrated with a series of ethanol and with acetone. They were embedded in Quetol 653 mixture (Nisshin E.M., Tokyo). Triphenyl antimony was added to the mixture at 0.5 M as an internal standard for section thickness [18]. After the resin was polymerized at 80°C, both semi- and ultrathin sections were cut on an LKB 8800 Ultratome III with glass knives. Median semithin sections, 1 μm, thick, were cut from the resin-embedded slice perpendicularly to its surface, dried and mounted in Biolite. These semithin sections measured 38 μm in distance from the top to the bottom surface probably due to shrinkage during the preparation process.

Ultrathin sections for quantitation by EDAX

Median transverse ultrathin sections of uniform silver-gold interference color were cut from the resin-embedded slice in parallel with its surface, and they served for morphometric and EDAX measurements. Special care was taken to include both the peripheral triad region and the central vein of a lobule in the ultrathin sections concerned. These sections, without being subjected to any additional electron staining, were put on a formvar-coated copper grid of 200 mesh for ultrastructural examination, and on a formvar-coated nylon grid of 180 mesh for EDAX measurements. They were dried and again coated with carbon by evaporation. A JEM 100 C electron microscope was used at the acceleration voltage of 80 KV for the former purpose, and a JEM 1200 EX electron microscope fitted with an energy dispersive X-ray analyzer (TN-2000, Tracor Northern Inc., Middleton) was used for EDAX analysis at the acceleration voltage of 100 KV and the beam current of 80 μA. The spot pulse analysis was made at an assigned point of a stained mitochondrion and its adjacent cytoplasm to obtain the local distribution of platinum under 60,000 × magnification for 500 sec. For quantitation of the oxidase activity, energy scanning pulse
Figs. 1-6.
analysis was carried out over a measuring field of 0.83 $\times$ 0.83 $\mu$m² covering an appropriate part of a Pt-DAB stained mitochondrion, and that covering its adjacent cytoplasm under 20,000 $\times$ magnification for 500 sec, and the net counts for $M_0$ of Pt (1.90–2.22 KeV) were obtained. The specific counts were calculated by subtracting the net counts for the cytoplasm from those for the mitochondrion. Before and after the X-ray analysis, electron micrographs of the specimen were taken for morphometry. The area of the positive site (AR) in the mitochondrion concerned was estimated with an image analyzer (Shōwa Denkō, Tokyo) by point count method under adequate thresholds. The section thickness variation was checked by examining the net counts for Lal (3.48–3.74 KeV) of Sb internal standard in each measurement, and the mean counts of 100.2 $\pm$ 15.1 per 500 sec were obtained. Assuming that the reactive site configuration was similar along the section depth, the oxidative activity (OA) for an individual mitochondrion was calculated with the correction for thickness variation as follows: OA = ($\frac{\text{specific counts for } M_0 \text{ of combined Pt in each mitochondrion}}{\text{(AR in } \mu \text{m}^2 \text{ of the reactive site concerned)}} \times (\text{mean Lal counts of Sb internal standard})^{(\text{Lal counts of the standard in each measurement})}$). The daily counting variation was also checked by counting an ultrathin section of Quetol resin containing tetrakis (triphenylphosphine) platinum (Fluka A. G., Buchs) at 0.1 M [18]. This deviation was within 5%.

III. Results

The cytochemical features of Pt-DAB reaction as evaluated by microspectrophotometry

When mouse hepatocytes were subjected to the Pt-DAB reaction for cytochrome oxidase, numbers of mitochondria were stained as solid black rods or filaments in the cytoplasm. They were the only site of positive reaction in these cells. In semithin sections, an individual mitochondrion was clearly visible with sharp contour (Fig. 1). No black staining of mitochondrria was recognizable at all in the sections treated with the three kinds of blank reagents mentioned above.

The black reaction product due to the Pt-DAB reaction gave a broad absorption peak at 500 nm (Fig. 2). The reaction began soon after the section was immersed in the reagent, and proceeded linearly from 5 to 45 min at 25°C and 37°C (Fig. 3).

Some parameters for the Pt-DAB reaction were check- ed (Figs. 4, 5). The reaction was maximal at pH 7.4, at 37°C and at 5 mM for DAB and at 1.0 mg/ml for cytochrome so far tested. The suboptimal temperature of 25°C and a reduced concentration of 2.5 mM for DAB and that of 0.25 mg/ml for cytochrome were recommended, because both high temperature and high cytochrome concentration accelerated the formation of non-specific precipitations, and because high DAB concentration made the pH adjustment of the reagent difficult. A linear relationship existed between the color intensity and the
Figs. 7–16.
logarithm of Pt to DAB concentration ratio of the reagents used. The addition of catalase at 0.1 and at 0.2 mg/ml brought about no recognizable change in reaction intensity. The section thickness permitting even penetration of the reagent during 45 min of immersion at 25°C was 40 μm at the maximum. The reaction product was stable for a month at 4°C and at 37°C in the dark, but faded slightly (5%) at 37°C under continuous illumination of a fluorescent lamp at 200 erg·cm⁻² per sec.

The reaction was inhibited by the presence of KCN and NaN₃ during the incubation and by the heat pretreatment of the sections. Potassium cyanide caused 49.9% inhibition at the final concentration of 0.1 mM, 88.3% at 1 mM and 91.9% at 10 mM. The presence of NaN₃ brought about 44.5% inhibition at 1 mM, 77.3% at 10 mM and 87.4% at 100 mM. An inhibition of 92.3% was obtained by heat treatment at 80°C for 15 min. The Lineweaver-Burk’s double reciprocal plot of the reaction rates against the DAB concentrations of the reagents used gave linear relationships at 25°C and 37°C (Fig. 6). The rates were expressed by the increase in optical density at 500 nm per min during the linear period of reaction from 10 to 30 min, and the external DAB concentrations by mM [11]. Thus, this plot revealed that the reaction conformed to Michaelis-Menten’s formula [15].

**Solubility of the reaction product**

The solubility in some organic solvents of the Pt-DAB reaction product produced in liver sections via cytochrome oxidase was examined. The product was insoluble in ethanol, acetone-NSA mixture and MEK. It showed a poor solubility of 0.04 in acetone, 0.12 in DMFA and 0.18 in DMSO.

**Reagent penetration into non-frozen specimens**

The local reaction intensity was measured by microspectrophotometry in ten assigned cytoplasmic fields. These fields were arranged in a row along an axis perpendicular to the section surface at 2 μm intervals. Each of them was scanned in parallel with the section surface. The intensity was shown by relative values, assuming the value of the fifth middle field as 1.00. To give a typical intensity distribution, the 1st top field, 1.51; the 2nd one, 1.05; the 3rd one, 0.98; the 4th one, 1.01; the 5th one, 1.00; the 6th one, 0.99; the 7th one, 1.01; the 8th one, 0.99; the 9th one, 1.04 and the 10th bottom one, 1.46. This proved that the reagent penetration into the liver slice during 45 min of reaction at 25°C was complete enough to permit the EDAX quantitation [cf. 10], though some unspecific staining occurred on the section surfaces.

**Ultrastructural development of Pt-DAB reaction product**

The local density development due to the Pt-DAB reaction in mitochondria was followed in sequence [cf. 5]. At 15 min of reaction, an increase in density was detected along the inner and the cristal membrane, and some of the intracristal spaces were found dense due to the deposit of the reaction product (Fig. 7). The intracristal spaces of high electron density increased in number per mitochondrion, and some parts of the peripheral spaces became dense at 30 min (Fig. 8). At 45 min of reaction, most of the intracristal and the peripheral spaces were occupied by the dense Pt-DAB reaction product (Fig. 9).

Mitochondria were clearly visible in the specimens treated with the blank reagent deprived of Pt at 45 min of reaction without postosmification. The peripheral and the intracristal spaces were fairly dense due to the DAB oxidation product (Fig. 10). The local distribution of this product in mitochondria coincided with that of the Pt-DAB reaction product, though the electron density of the former product was far less than that of the latter. In contrast, mitochondria were not discernible from the cytoplasmic matrix in the specimens treated with the blank reagent deprived of DAB (Fig. 11) and treated with that deprived of both Pt and DAB (Fig. 12). They were visible merely as elliptic spaces surrounded by the cisternae of the endoplasmic reticulum, their membranous structure being indistinguishable entirely.

**Local distribution of Pt-containing product in mitochondria**

Preliminary examinations proved the existence of the Pt-containing reaction product in a single Pt-DAB treated mitochondrion by the presence of prominent M₄ and L₅ peaks of Pt in the energy dispersive X-ray spectrum (vid. Fig. 3 of reference 7). As the first access to quantitative analyses, the stability of the Pt-DAB reaction product against electron beam irradiation was checked. The pulse counting for M₄ peak of Pt bound to Pt-DAB stained mitochondria was made at every 100 sec interval for 500 sec. Taking the counts during the first interval as 1.00, those during the second interval were 1.00±0.15, those during the third interval 0.97±0.13, those during the fourth interval 0.98±0.13 and those during the fifth interval 0.98±0.10 (means of 15 estimations). This showed that the product was practically stable against 500 sec of irradiation. Similar stability was proved for triphenyl antimony by checking pulse counts for L₅ peak of Sb.

The spot pulse analysis revealed the local counts for M₄ peak of Pt as follows: 499.1±64.9 for the peripheral space, 608.8±84.4 for the intracristal space, 60.6±10.9 for the mitochondrial matrix and 72.0±14.4 for the adjac-

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1) For comparison’s sake, the solubility was checked for the DAB reaction product and its postosmificated one in situ (vid. explanation of Fig. 2 for the postosmification procedure). The former product showed the solubility of 0.15 in MEK, 0.26 in ethanol, 0.42 in acetone-NSA mixture, 0.63 in acetone, 1.04 in DMFA and 2.43 in DMSO, while the latter the solubility of 0.04 in MEK, 0.09 in ethanol, 0.15 in acetone-NSA mixture, 0.28 in acetone, 0.64 in DMFA and 0.94 in DMSO.
cent cytoplasmic matrix (means of 5 estimations). This result proved that the local distribution of Pt coincided with that of the Pt-DAB reaction product. The information obtained from the image pulse analysis also supported this view (vid. Fig. 2 of reference 7). In contrast, mitochondria gave the local counts of 15.7±3.5 in the specimen treated with the Pt medium (mean of 5 estimations), probably due to diffuse adsorption. Needless to say, they showed no count at all in the specimens treated with the DAB reagent and with the basal medium.

**Cytochrome oxidase activity in hepatocytes of different location within a lobule**

A gradient in cytochrome oxidase activity was demonstrated by the Pt-DAB reaction along the radial axis of a liver lobule through the peripheral region (PE) adjacent to the triad and the central region (CN) close to the central vein (Fig. 13)\(^3\). Some morphometric differences in hepatocyte mitochondria were found between these two regions \(\text{[cf. 12]}\). The hepatocytes in the PE showed 20.3±2.1 mitochondria per 100 μm\(^2\) cytoplasmic area (Fig. 14), while those in the CN 30.2±2.3 (Fig. 15). The area of the Pt-DAB reactive site covered by the EDAX measuring field was 0.085±0.029 μm\(^2\) in the PE mitochondria, while 0.058±0.021 μm\(^2\) in the CN ones.

The oxidase activity was quantitated at individual mitochondrial level, to get a better understanding of this gradient. As the maximum activity found was 3,396 counts per μm\(^2\) of the reactive site per 500 sec per mean thickness, the activity of each mitochondrion was shown by the relative value, assuming this maximum value as 100.0. The quantitation was carried out for random 66 pairs of mitochondria in these two regions, PE and CN, and the relative activity for each mitochondrion was given in Fig. 16. From this activity distribution, the OA for the PE mitochondria was calculated to be 63.0±15.0 while that for the CN ones 42.0±15.0. Namely, the former OA was 1.5 times as strong as the latter one. The analysis of variance proved that this activity difference between the PE and the CN mitochondria was statistically significant.

**IV. Discussion**

**Cytochemical features of Pt-DAB reaction**

In view of the facts that the Pt-DAB reaction conformed to the Michaelis-Menten's formula, that it was inhibited sensitively by KCN, NaN\(_3\), and heat, and that its reaction product showed the same local distribution as the DAB oxidation product did, it is no other than the DAB polymerization reaction via cytochrome oxidase going hand in hand with the concurrent incorporation of Pt atoms. This Pt incorporation makes the reaction product large in physical\(^1\) and in electron density, extremely poor in solubility in various organic solvents, and stable against electron beam irradiation. All these features make the Pt-DAB reaction suitable for EDAX quantitation of cytochrome oxidase at electron microscopic level \([\text{cf. 4]}\).

**Gradient of cytochrome oxidase activity in liver lobule**

A gradient in cytochrome oxidase activity has been reported to exist along the radial axis of a liver lobule from the PE to the CN in varieties of rodent species \([1, 9, 14, 20]\). In the present study, the local activity difference was quantified between the PE and the CN at individual mitochondrial level. The mitochondria in the PE hepatocyte were 1.5 times stronger in activity per unit area of the reactive site than those in the CN one. A stereological morphometric analysis of mitochondria in rat liver cells has been reported in detail by Loud \([12]\). A comparison of his result with our morphometric one leads us to the supposition that the mitochondrial population and the reactive site configuration are similar in these two rodent species. Based on this, the relative oxidase activity per cell in the PE and the CN was calculated.

According to Loud \([12]\), the mitochondrial number per cell was 1,060 for the PE hepatocyte while 1,600 for the CN one, and the reactive site, including both the peripheral and the intracristal space, was 37.9 μm\(^2\) in area for the average PE mitochondrion while 20.2 μm\(^2\) for the CN one. The relative cytochrome oxidase activity per unit area of the reactive site was 1.5 for the PE mitochondrion while 1.0 for the CN one. By multiplication of these three parameter values, the OA was calculated to be 60,261 for the average PE hepatocyte while 32,320 for the average CN one. In other words, the OA in the former hepatocyte was estimated to be 1.9 times stronger than that in the latter one. As the average cytoplasmic volume was the same in both the PE and the CN hepatocyte \([12]\), the activity ratio of the former cell to the latter one was 1.9 : 1.0 per cytoplasmic volume. A microspectrophotometric estimation at cellular level in a 10 μm thick mouse liver section proved the oxidase gradient in a lobule (vid. Fig. 9 of reference 9). Taking three hepatocytes lying on the triad side as the PE cells and those lying on the central vein side as the CN ones, the mean activity expressed by the chromophore amounts per 1 μm\(^2\) cytoplasmic area or 10 μm\(^3\) volume was 10.2 for the former cells while 5.4 for the latter ones. This again gave the activity ratio of 1.9 : 1.0. The estimation at the mitochondrial and that at the cellular level coincide satisfactory with each other.

\(^3\) This gradient was proved to exist even when the perfusion was carried out from the inferior vena cava to the portal vein.

\(^1\) The physical density of the purified reaction products obtained via horseradish peroxidase and hydrogen peroxide \([6]\) was measured by the floating method in heavy liquid, a mixture of methylene iodide and benzene. The Pt-DAB reaction product showed the density of 2.156 at 20°C. The DAB reaction product gave the density of 1.474, and that of 2.064 after postosmification.
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VI. References


