Isolation and Properties of Human Platelet Mitochondria

By Miriam H. Fukami and Leon Salganicoff

A mitochondrial fraction of human platelets that exhibited respiration coupled to ADP utilization has been isolated. These activities were sensitive to classical inhibitors of mitochondrial respiration and oxidative phosphorylation. Pyridine nucleotide-linked substrates were oxidized at low rates (4–7 natoms/min/mg), but succinate and α-glycerophosphate were oxidized more rapidly (16 and 38 natoms/min/mg, respectively). Difference spectra showed the presence of the mitochondrial respiratory chain cytochromes, c_1 + c, b, and a + a_3. Electron micrographs revealed the presence of intact mitochondria with well-preserved outer membranes, as well as various α-granules. There appeared to be no qualitative differences between these human platelet mitochondria and pig platelet mitochondria isolated by the same procedure.

THE ROLE OF oxidative phosphorylation in over-all platelet energy metabolism has been undergoing reevaluation in recent years. Early investigators considered that glycolysis was the main pathway for energy metabolism in platelets.[1-3] In fact the competence of platelet mitochondria to carry out oxidative phosphorylation was in question at one time.[3,4] More recently, it has been shown by various laboratories that respiration in intact platelets is coupled to phosphorylation.[5,6] In order to evaluate the importance of the oxidative pathway, a characterization of platelet mitochondria was undertaken in this laboratory. Although a number of workers have reported the isolation of subcellular fractions from human platelets which contained intact mitochondria, they either did not examine the properties of the mitochondria or found that they were uncoupled.[3,7-9]

A method for the isolation of coupled platelet mitochondria was developed first with pig platelets because they are readily available in large quantities.[10] Coupled human platelet mitochondria have now been isolated by the same technique and characterized. Their properties are strikingly similar to those of pig platelets, although significant species differences seem to exist for some platelet reactions.[11]

MATERIALS AND METHODS

The technique used for the preparation of the human platelet subcellular particles was essentially the same as that developed for the pig platelet preparation.[10] Ten units of platelet-

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rich plasma (PRP) obtained from a blood bank within 2 3 hr after the blood was drawn from donors were centrifuged at 1000 *g* for 10 min to provide the platelets used for these preparations. The white cell contamination of the PRP was usually less than 3/cu mm. The unwashed platelets were suspended in approximately 20 ml of ice-cold medium consisting of 100 m*M* KCl, 50 m*M* Tris chloride, pH 7.4, 5 m*M* $MgCl_2$ and 1 m*M* EDTA and incubated with 15 mg of a proteinase (Nagarse)[12] for 5 min without further cooling. The platelets were removed from the Nagarse by centrifugation and resuspended in fresh, cold medium of the same composition supplemented with 1 m*M* ATP and 0.35% bovine serum albumin. All subsequent procedures were carried out at 4°C. The nagarse-treated platelets were next subjected to two disruption cycles through a modified French pressure cell[10] at 800 psi. In order to avoid subjecting the subcellular components released during the first breakage to additional pressure, the suspension from the first disruption was centrifuged at 1000 *g* for 10 min and the pellet resuspended in fresh medium for the second cycle. The suspension from the second breakage was also centrifuged at 1000 *g* for 10 min, and the residue was washed once with fresh medium. After another centrifugation at 1000 *g* for 10 min, the combined supernatant fractions were centrifuged at 12,000 *g* for 10 min to yield the crude mitochondrial preparation. This pellet was washed twice with fresh medium and suspended in 250 m*M* sucrose buffered with 10 m*M* Tris chloride, pH 7.4, at a final concentration of approximately 25 mg/ml.

Electron micrographs were kindly prepared by Dr. G. Stewart of the Specialized Center of Research in Thrombosis.

Nagarse was obtained from the Enzyme Development Corporation, New York, N.Y. All other chemicals were of the highest commercial grade available.

RESULTS AND DISCUSSION

Electron micrographs of the preparation showed the presence of well-preserved mitochondria with complete outer membranes (Fig. 1). Membrane fragments, cell debris, actomyosin fibers, and α-granules with different staining

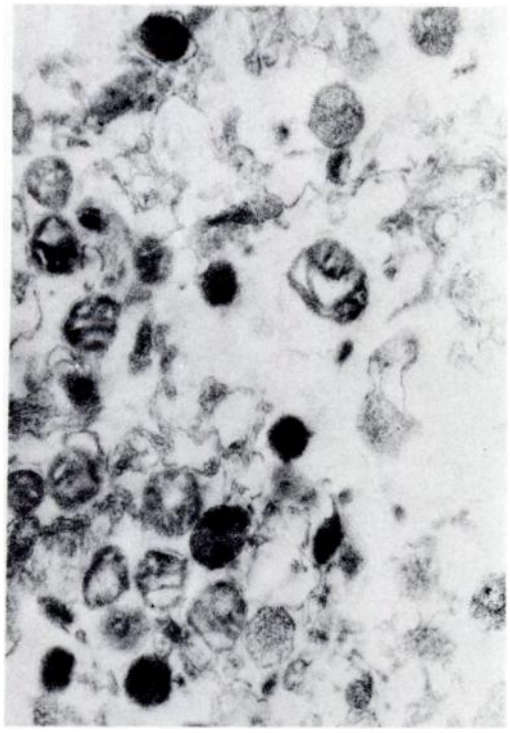

Fig. 1. Electron micrograph of the platelet organelle preparation. The pellet was suspended in 5% glutaraldehyde in cacodylate buffer (pH 7.4, 350 mOsM) and fixed in the cold for 30 min. The subcellular material was sedimented by centrifugation at 12,000 g for 10 min. The pellet was rinsed briefly with cacodylate buffer and fixed with 1% osmium tetroxide in the cold for 1 hr. The osmium was rinsed out with buffer, the sample dehydrated with ethanol and embedded in Epon 812. Sections were double stained with uranyl acetate followed by lead citrate and micrographed at an original magnification of 52,000 ×.

Table 1. Human Platelet Mitochondria Respiration Rates

Substrates	R.C.	P.O.	natoms/min/mg
α-ketoglutarate	5.9	3.4	3.8–6.7
Pyruvate-malate	3.5	2.5	4.0–6.6
Succinate	2.1	2.1	10–16.2
α-glycerophosphate	1.8	1.9	26.9–38.8
Ascorbate-TMPD +	1	—	100–124

Oxygen consumption was measured at 23–27°C in a stirred, 1-ml Lucite chamber fitted with a Clark-type electrode and a pH meter. The reaction medium consisted of a 0.25 M sucrose, 10 mM Tris chloride, and 10 mM potassium phosphate, both pH 7.4. Approximately 1–2 mg of protein was used per reaction with 5–10 mM substrate. State three was induced by the addition of 150 nmoles of ADP. The results shown are the average of three determinations.

characteristics were also seen. The presence of nonmitochondrial material did not seem to interfere with either mitochondrial function or spectral characterization except by diluting specific activities. Further purification of the preparation was not attempted since our experience with pig platelet particles had shown that phosphorylating activity was lost with gradient fractionation and multiple washings. The state-3 or ADP-stimulated respiration rates, P : O ratios, and respiratory control ratios, i.e., the ratio of the ADP-stimulated rate to the ADP-depleted rate,[13] of platelet mitochondria oxidizing various substrates are shown in Table 1. Exogenous NADH was not oxidized by this preparation, indicating that the mitochondrial membranes were intact. Oxidation of pyridine nucleotide-linked substrates such as α-ketoglutarate and pyruvate was slower than that of succinate and α-glycerophosphate. The P : O ratios observed for the different substrates were similar to those found in other mammalian mitochondria, approximately two for succinate and α-glycerophosphate, more than two for pyruvate and malate, and more than three for α-ketoglutarate. The oxidation of α-ketoglutarate and pyruvate was sensitive to inhibition by rotenone (1 μ*M*), and the oxidation of succinate and α-glycerophosphate was inhibited by antimycin A (5 μg). Inhibited respiration in antimycin A-treated mitochondria was stimulated by the addition of ascorbate and

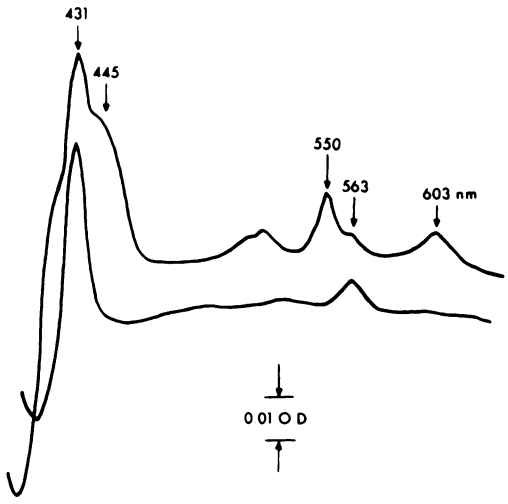

Fig. 2. Room-temperature spectra of mitochondrial cytochromes. Difference spectra were obtained with a scanning single-wavelength double-beam spectrophotometer[12] with approximately 6–10 mg of protein/cuvette. The reference cuvette was left without added substrate, and α-glycerophosphate was added to the measuring cuvette. The preparation was allowed to go anaerobic in the upper trace, while antimycin A was added to the measuring cuvette in the lower trace.

Table 2. Cytochrome Content of Human Platelet Mitochondria

Cytochrome	Concentration (nmoles/mg protein)	Component Cytochrome, a, a_3
a, a_3	0.080	1.00
b	0.044	0.55
c_1, c	0.081	1.01

The concentration of cytochromes were calculated from the spectra shown in Fig. 2 using the extinction coefficients given by Chance.[14]

N,N,N',N'-tetramethyl-*p*-phenylenediamine (TMPD). The effect of these respiratory inhibitors indicated that the organization of the electron transport chain was the same as that found in other mitochondria.

Difference spectra of anaerobic versus aerobic preparations confirmed the presence of the classical respiratory carriers (Fig. 2).[14] The upper trace shows the α-bands of cytochromes a + a_3, b, and c_1 + c at 603, 563, 550 nm, respectively; the peaks in the Soret region appeared somewhat out of proportion, particularly in the 430-nm region, probably due to some hemoglobin contamination. The lower trace shows the difference spectrum of antimycin A-treated and aerobic preparations, i.e., the spectrum of cytochrome b. The cytochrome contents and their stoichimetries as calculated from these spectra are given in Table 2. The significant excess of cytochromes $c(c_1)$ which is seen in other types of mitochondria such as rat liver was not apparent here.

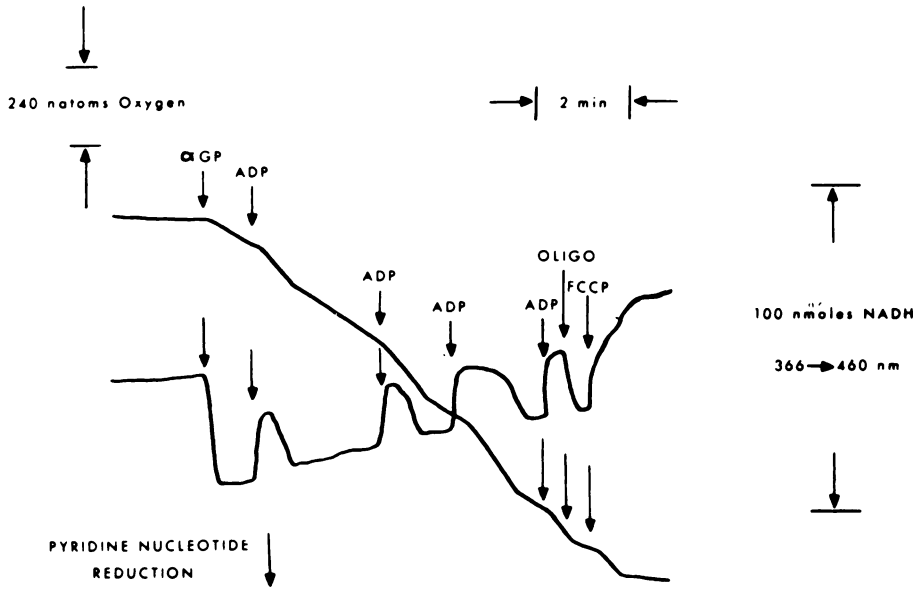

Fig. 3. Pyridine nucleotide oxidation-reduction changes accompanying various mitochondrial respiratory states. The reaction conditions were as described for Table 1 except that a 2.5-ml chamber was used with 5 mg of protein. Pyridine nucleotide fluorescence and oxygen consumption were followed simultaneously; the excitation wavelength was 366 nm, and the emission was measured at 460 nm. Additions were made to the reaction as follows: 10 μmoles of α-glycerophosphate (αGP), 250 nmoles ADP, 250 nmoles ADP, 500 nmoles ADP, 500 nmoles ADP, 5μg oligomycin, and 1 nmole FCCP.

The coupling of oxidative phosphorylation to respiration was demonstrated both polarographically and by pyridine nucleotide oxidation-reduction changes monitored fluorimetrically as shown in Fig. 3. The resting mitochondrial preparation appeared to be substrate-free and did not respire without addition of exogenous substrate. When α-glycerophosphate was added, oxygen consumption was initiated and pyridine nucleotides became reduced, due to energy-linked reversal of electron transport.[15] Repeated additions of ADP resulted in cycles both of stimulation in respiration and of oxidation of pyridine nucleotides. When oligomycin (5 μg) was added to the reaction in the middle of an ADP cycle, respiration was slowed to the rate observed in the absence of ADP, and pyridine nucleotides became reduced. This inhibition of coupled respiration was relieved by the addition of an uncoupler, carbonylcyanide-*p*-trifluoromethoxyphenyl-hydrazone (FCCP), which caused increased oxygen uptake and oxidation of pyridine nucleotides.

CONCLUSION

Although it is now established that oxidative phosphorylation does occur in platelet mitochondria, the significance of the oxidative pathway in platelet energy metabolism is still difficult to evaluate. The number of mitochondria per platelet is extremely low relative to the high levels of glycolytic enzymes in platelets,[1] and the rate of platelet respiration is fairly low. If most of the oxygen consumed by platelets were utilized via the citric acid cycle and the electron transport chain in the mitochondria, oxidative phosphorylation would account for well over half of all platelet ATP because of the greater efficiency of oxidative phosphorylation compared to glycolysis. However, none of the dehydrogenases leading to or in the citric acid cycle appeared to be especially active in this preparation. α-glycerophosphate oxidation may not be significant, since the levels of the cytosolic dehydrogenase appear to be too low[1,16] to be able to provide enough substrate for the oxidase. Also there are indications that pathways for oxygen utilization other than the respiratory chain may exist.[17,18] Oxidative phosphorylation appears to be essential in platelets under special circumstances,[5,19] but under normal conditions (in the presence of glucose), both glycolysis and oxidative phosphorylation seem to be able to compensate for diminished activity in the other with respect to such functions as aggregation, the release reaction, and clot retraction.[6,11,18]

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