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Mitochondrial swelling induced by Ca2+ and inorganic phosphate and its related phenomena

Kozo Utsumi*

*Okayama University,

Mitochondrial swelling induced by Ca2+ and inorganic phosphate and its related phenomena*

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Abstract

Some investigations have been done on the relationships between the swelling-shrinkage change, oxygen consumption and state of oxidation-reduction of pyridine nucleotides of mitochondria, and between the swelling-shrinkage change of mitochondrial structure by Ca2+ and accumlation of Ca45 in rat liver mitochondria. A parallel relationship is observed between the Ca2+ induced swelling and Ca2+ accumulation. Both of them require Pi but not Mg2+, ATP and exogenous respiratory substrates and are inhibited by respiratory inhihitors or uncouplers of oxidative phosphorylation but not by the inhibitors of phosphorylating respiration. In this case the Ca2+ is transported with the phosphate even in ice cold. Even in the presence of antimycin A, moreover, Pi-dependent Ca2+ accumulation and Ca2+ induced swelling can be overcome by addition of ATP, which are inhibited by oligomycin. In the presence of Pi, mitochondria show shrinkage by addition of Ca2+ before the high amplitude swelling, which is closely correlated to the electron ransport chain and phosphorylation process of mitochondria, and the pattern of the mitochondrial shrinkage is quite similar to that observed in the case of respiratory control by ADP in intact mitochondria. This shrinkage of mitochondria is inhibited by respiratory inhibitor or uncoupler of oxidative phosphorylation but not by the inhibitor of phosphorylating respiration. From these data, therefore, it is considerd that the Ca2+ accumulation and Ca2+ induced shrinkage-swelling of mitochondria require the energy of oxidative phosphorylation with respect to the initial step before the oligomycin block.

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MITOCHONDRIAL SWELLING INDUCED BY Ca^{2+} AND INORGANIC PHOSPHATE AND ITS RELATED PHENOMENA

Kozo UTSUMI

Department of Biochemistry, Cancer Institute of Okayama University Medical School, Okayama, Japan

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Using turbidimetric and light-scattering techniques swelling-shrinkage of mitochondria have been investigated by many workers[1~8]. The relationship between the structural state and the light-scattering properties of mitochondria is not precisely understood, but the degree of mitochondrial swelling is proportional quantitatively to the intramitochondrial water content[1,8].

Recently, PACKER[9] described two types of swelling-shrinkage changes manifested by isolated mammalian heart mitochondria. One of them is "phase I" or "low amplitude" swelling-shrinkage (about 20-40 per cent change in mitochondrial volume) and the other is "phase II" or "high-amplitude". The former is brought about rapidly and reversibly by normal reactants of respiratory chain and phosphorylating system showing its dependency on the energy-coupling mechanism and/or respiration. Moreover, the phase I type of swelling was observed in the suspension of Ehrlich ascites tumor cells to be accompanied with the CRABTREE effect[10]. Namely, mitochondrial volume changes of this type reflect physiology of mitochondria.

Contrarily, phase II swelling-shrinkage seems to be only partly under control of the respiratory chain: mitochondrial function is decreased stepwise proportional to the swelling and the activity of respiratory control is lost.

Calcium ions (Ca^{2+}) induce the both types of swelling under a given special condition and elicit the uncoupling of oxidative phosphorylation of intact mitochondria but not of submitochondrial fragment[11,12]. Regarding the Ca^{2+}-induced swelling of mitochondria, WOJTCZAK and LEHNINGER suggest that Ca^{2+} accelerate mitochondrial swelling indirectly by accelerating the enzymic formation of U factor from a precursor lipid. In addition, PUMPHREY and REDFEARN[14], and UTSUMI[8] observed that the Ca^{2+}-induced swelling of mitochondria requires inorganic phosphate (Pi) in the medium of sucrose as in the case of Ca^{2+} accumulation in mitochondria. The Ca^{2+}-induced swelling of this type, however, is not prevented by bovine serum albumin (BSA). Therefore, it seems probable that the Ca^{2+}-induced swelling of mitochondria is associated with Ca^{2+} accumulation.

From these observations it seems that studies with Ca^{2+} would give a new

approach to clarify the relationships among mitochondrial swelling, ion accumulation, and oxidative phosphorylation of intact mitochondria.

This paper shows that the swelling of rat liver mitochondria induced by Ca^{2+} is brought about in association with Ca^{2+} accumulation aud ATP formation, and that the sweling and the Ca^{2+} accumulation are controlled by respiratory substrates, Pi, magnesium ions (Mg^{2+}), adenosine triphosphate (ATP) and by the concentration of Ca^{2+}.

MATERIALS AND METHODS

Preparative: Albino rats, weighing 150-200 g, fed on a semi-synthetic diet (Funahashi No. 20), were used for this investigation. They were killed by decapitation, the livers were immediately removed, weighed and transferred to ice cold sucrose solution (0.25 M). Mitochondria were isolated as previously described[8].

Adenosine 5′ diphosphate (ADP), ATP and antimycin A were obtained from Sigma Chemical Co. Tributyltin chloride (TBTC) was donated by Prof. HAGIHARA (University of Osaka) and oligomycin by Dr. MINAKAMI (University of Tokyo)

Measurements: The volume changes of mitochondria were studied by measuring the changes in 90° light-scattering at 650 mμ. The changes of mitochondria was proportional to those of 90° light-scattering at 650 mμ. The intensity of scattered light was recorded, the initial level was defined as 100 per cent and the percentage of changes in scattering obtained after addition of reagents recorded on chart. Oxygen consumption and oxidative phosphorylation were measured with rotating platinum electrode as described in previous paper[8,15]. Reduced pyridine nucleotides of mitochondria were excited at 365 mμ line of Hg lamp and the fluorescence was monitored at 450 mμ using a photomultiplier (1P21) positioned at 90° to the exciting light and recorded by autorecorder in arbitrary units or relative intensity. These oxygraphic-, fluorescence-and 90° light-scattering-traces were carried out simultaneously by using the apparatus reported in previous paper[15].

Basic incubation mixture was consisted of 0.2 mmoles sucrsoe, 40 μmoles KCl, 10 μmoles Tris-HCl buffer (pH 7.4), 2 μmoles $MgCl_2$ and 80 mμmoles ethylenediaminetetraacetic acid (EDTA) in final volume of 2 ml. The incubation was carried out at 25° and the detail of each experimental conditions is given in the legends to figures.

Translocation of inorganic phosphate and calcium ions: The translocation of P^{32} and Ca^{45} into rat liver mitochondria was measured by the method of AZZONE and ERNSTER[16]. For the translocation of P^{32}, mitochondria equivalent of

3-5 mg protein were incubated in 3 ml of the medium containing 0.3 mmoles sucrose, 60 μmoles KCl, 30 μmoles Tris-HCl buffer (pH 7～4), 9 μmoles Na-succinate or α-ketoglutarate (α-KG), 3～30 μmoles $MgCl_2$, 0.3-3 μmoles $CaCl_2$, 120 mμmoles EDTA, and 9 μmoles phosphate buffer (pH 7. 4 containing about 10 μc P^{32}). For the translocation of Ca^{45}, K-phosphate containing P^{32} and $CaCl_2$ in the medium for test of P^{32} translocation were replaced with non-labelled K-phospnate and $Ca^{45}Cl_2$ (about 10～30 μc). The incubations were carried out at 0-25° in Taiyo metabolic shaker with air as the gas phase. After 2-minutes equilibration period, P^{32} or Ca^{45} was added to the flasks. At an indicated time 1 ml aliquot of the reaction mixture was removed and rapidly filtered by suction through a pad of celite by the method of SALLIS *et al*[17]. Celite retained mitochondria were washed twice with 1 ml portions of ice cold incubation mixture (devoid of Pi and calcium) and the mitochondria retained on the pad were extracted with 8 per cent perchrolic acid for measurement of Pi or dried at 110° for one hour for the counting of translocated Ca^{45}. The counting of P^{32} and the quantitative determination of Pi were carried out on the Pi fraction of TAKAHASHI's method[18]. Mitochondrial protein was determined by the method of KJELDAHL[19].

RESULTS

Mitochondrial swelling by calcium ion without exogenous substrates : As shown in Fig. 1, addition of Pi (3 mM) to the basic incubation mixture resulted in a slightly increase in the oxygen consumption, swelling and oxidation of pyridine nucleotides. On addition of Ca^{2+}(0.1 mM) there occurred simultaneously a rapid increase of 90° light-scattering (2—8 per cent), though being transient, an increase in oxygen uptake and a marked oxidation of pyridine nucleotides with in a short time. Then the 90° light scattering of mitochondrial suspension was reduced to 40—50 per cent (so called high amplitude swelling) accompanied with the brake of respiration. In this instance, the concention of Ca^{2+} was insufficient to cause a high amplitude swelling without Pi as shown in Fig. 2. But in the absence of Pi, 0.1 mM Ca^{2+} reduced the fluorescence intensity transiently and increased it gradually depending on the time of incubation. Next, by addition of Pi (3 mM) after the incubation with Ca^{2+} the mitochondria showed a shrinkage (about 4—8 per cent) accompanied with the initial burst of respiration and with decrease of the fluorescence, and then turned to high amplitude swelling with the brake of respiration and with a further decrease of the fluorescence (Fig. 2). This high amplitude swelling of mitochondria induced by Ca^{2+} (0. 1 mM) in the presence of Pi was inhibited or reduced by respiratory inhibitors, amytal (1 mM), antimycin A (2 γ/ml) and KCN (1 mM), and by uncoup-

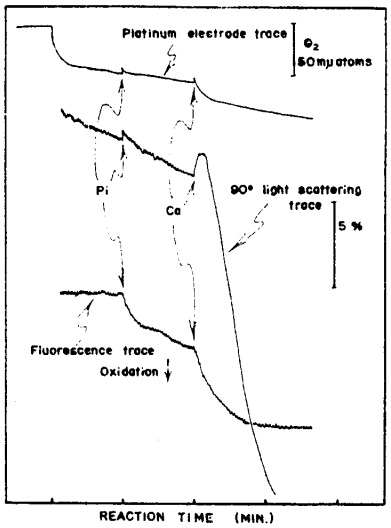

Fig. 1. Effee of Ca^{2+} on the swelling shrinkage changes and electron transport system of mitochondria. About 2.5 mg protein of rat liver mitochondria incubated in 2 ml of 0.1 M sucrose containing 40 μmoles KCl, 10 μmoles Tris-HCl buffer (pH 7.4), 2 μmoles $MgCl_2$ and 80 mμmoles EDTA at 25°. Platinum electrode trace refers to the oxygen concentration in the medium, time moves from left to right and downward deflection indicated oxygen consumption. 90° light-scattering trace refers to the swelling-shrinkage by 90° light-scattering and downward deflection indicates swelling. Fluorescence trace refers to the oxidation-reduction of pyridine nucleotides of mitochondria and downward deflection indicates oxidation. Arrows show the addition of substances. The amounts of additions were 6 μmoles Pi and 0.2 μmoles Ca^{2+}.

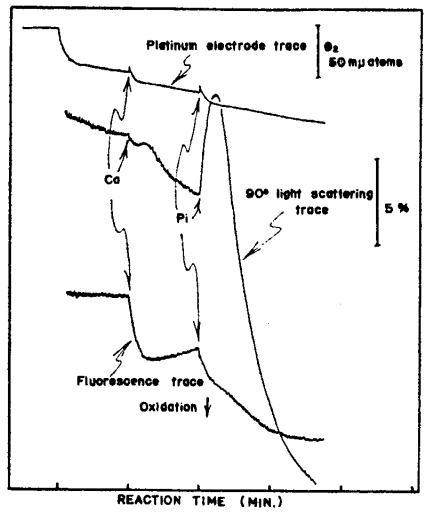

Fig. 2. Requiremnt of Pi for the Ca^{2+} induced swelling shrinkage of mitochondria. The experimental conditions ware same as in Fig. 1.

lers of oxidative phosphorylation such as 2,4-dinitrophenol (DNP 30 μM) and oleic acid (0.2 mM) but not by inhibitors of phosphorylating respiation, oligomycin (2.8 γ/ml) and TBTC (0.3 μM) as shown in Figs. 3, 4, 5 and 6. Then in the presence of DNP and Pi, the high amplitude swelling was not observed by adding Ca^{2+} but was by addition of respiratory substrates such as α-KG, glutamate, β-hydroxybutylate (β-OH) and succinate. The high amplitude swelling induced by Ca^{2+} was observed by addition of ATP (1 mM) even in the presence of antimycin A as shown in Fig. 4. This type of swelling was inhibited by oligomycin or TBTC.

Ca^{2+} induced mitochondrial swelling in the presence of succinate : As illustrated in the preceding paragaph, the Ca^{2+} induced swelling requires the electron transport and Pi, but the degree and pattern of swelling were different when succinate served as substrate from that of swelling in the presence of

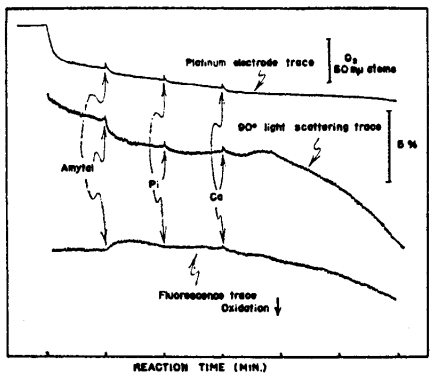

Fig. 3. Effect of amytal on the swelling-shrinkage changes induced by Pi and Ca^{2+}. The concentration of amytal was 2 μmoles and the other conditions were same as in Fig. 1.

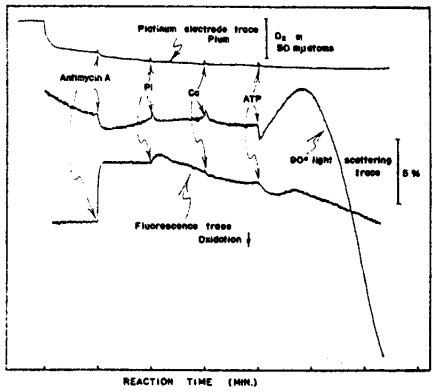

Fig. 4. Effect of antimycin A and ATP on the swelling-shrinkage changes of mitochondria induced by Pi and Ca^{2+}. The concentration of antimycin A and ATP were 2 γ and 2 μmoles respectively. Other conditions were same as in Fig. 1.

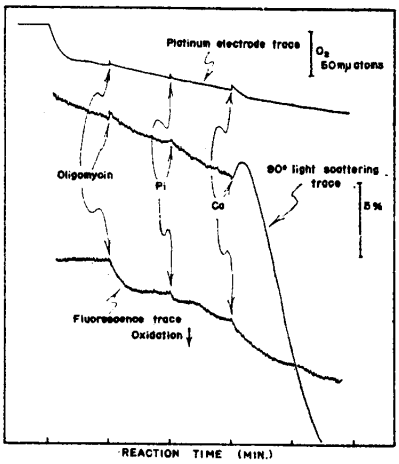

Fig. 5. Effect of oligomycin on the swelling-shrinkage changes of mitochondria induced by Pi and Ca^{2+}. The amount of oligomycin was 1.4γ and the other conditions were same as in Fig. 1.

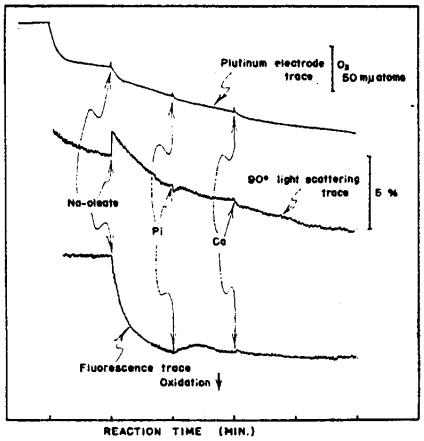

Fig. 6. Effect of Na-oleate on the swelling-shrinkage changes of mitochondria induced by Pi and Ca^{2+}. The concentration of Na-oleate was 0.1 μmoles and the other conditions were as in Fig. 1.

nicotinamide adenine dinucleotide linked substrate, e. g. β-OH, α-KG and glutamate. In the presence of pyridine nucleotide linked substrate, the swelling pattern of mitochondria induced by Ca^{2+} was similar to that of endogenous substrate except the effect of true uncoupler but in the presence of succinate and Pi the degree of swelling induced by Ca^{2+} was about 5—10 per cent per minute

accompanied by rapid oxidation of pyridine nucleotides and by increased respiration. About 20 minutes after addition of Ca^{2+}, a remarkable increase in fluorescence intensity was brought about accompanied by the brake of oxygen uptake and a decrease in 90° light-scattering (2—3per cent per minute), and followed by the gradual increase of respiration (respiratory release), and decrease of light-scattering and of fluorescence intensity (Fig. 7). In this instance, a remarkable swelling was brought about in parallel with decrease in fluorescence intensity,

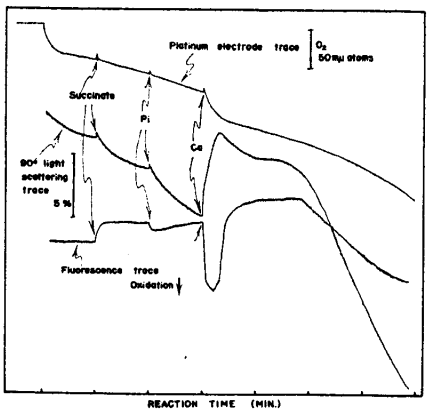

Fig. 7. Effect of Ca^{2+} on the swelling-shrinkage changes and electron transport system of mitochondria in the presence of Na-succinate. The concentration of Na-succinate was 6 μmoles and the other conditions were same as in Fig. 1.

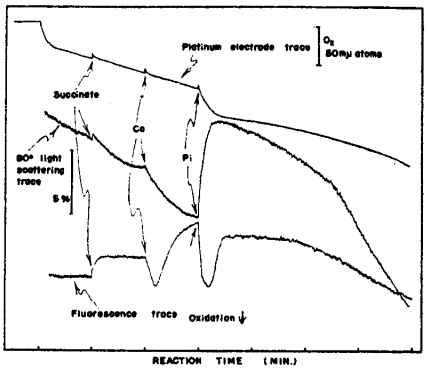

Fig. 8. Requirement of Pi for Ca^{2+} induced swelling-shrinkage changes of mitochondria in the presence of Na-succinate. The experimental conditions were same as in Fig. 7.

and the degree of swelling was about 40 per cent. These processes were changeable in the time period by the concentration of Ca^{2+} to mitochondrial protein and by the time of aging from preparation. In the presence of succinate, respiratory activity of mitochondria was not affected extensively by addition of Ca^{2+} but 90° light-scattering was slightly reduced and the fluorescence intensity was reduced transiently. This oxidation of pyridine nucleotides was brought about in 20 seconds and then an cxtensive reduction occurred again. By the addition of Pi an extensive increase of light scattering was induced simultaneously with the decrease in fluorescence intensity and with the increase in respiration, and followed by a remarkable increase of fluorescence intensity, brake of respiration and gentle decrease of 90° light-scattering. Then a gradual increase of respiration proceeded in parallel with the decrease in fluorescence and in 90° light-scattering as shown in Fig. 8. This Ca^{2+} induced swelling in the presence of succinate and Pi was inhibited by respiratory inhibitors, antimycin A, KCN, malonate (1 mM) + amytal (1 mM), but not by oligomycin and TBTC. Uncouplers of oxidative phos-

phorylation such as DNP (30 μM), amytal (1 mM) and oleic acid (0.1 mM) rather accelerated the swelling, and the shrinkage accompanied with an initial burst of respiration by addition of Ca^{2+} was abolished (Figs. 9, 10, 11, and 12). Fig. 12 shows a slight increase of respiration (respiratory release) by the addition of oleic acid and Ca^{2+}, but a remarkable respiratory release can be observed if succinate is added prior to the addition of oleate.

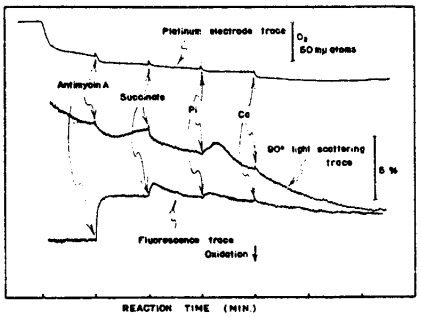

Fig. 9. Effect of amytal on the swelling-shrinkage changes induced by Pi and Ca^{2+} in the presence of Na-succinate. The concentration of amytal was 2 μmoles and the other conditions were same in Fig. 7.

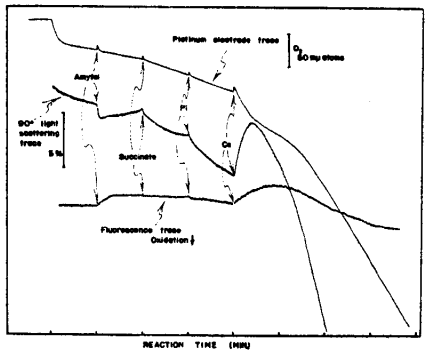

Fig. 10. Effect of antimycin A on the swelling-shrinkage changes of mitochondria induced by Pi and Ca^{2+} in the presence of Na-succinate. The amount of antimycin A was 2 γ and the other conditions were same as in Fig. 7.

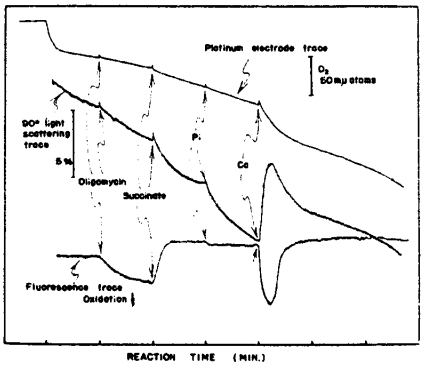

Fig. 11. Effect of oligomycin on the swelling-shrinkage changes of mitochondria induced by Pi and Ca^{2+} in the presenee of Na-succinate. The amount of oligomycin was 1.4 γ and the other conditions were same as in Fig. 7.

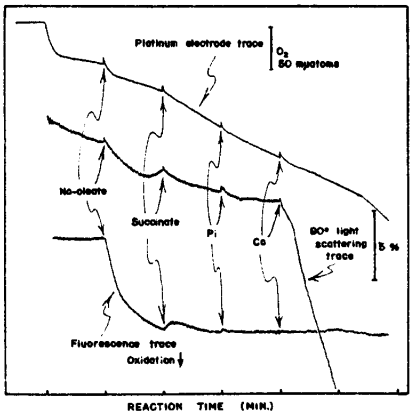

Fig. 12. Effect of Na-oleate on the swelling-shrinkage changes of mitochondria induced by Pi and Ca^{2+} in the presenee of Na-succinate. The concentration of Na-oleate was 0.1 μmole and the other conditions were same as in Fig. 7.

Accumulation of P^{32} and Ca^{45} in rat liver mitochondria in the medium containing 10 mM $MgCl_2$, 1 mM $CaCl_2$ and 3 mM ATP: Table 1 shows

Table 1. Accumulation of P^{32} in rat liver mitochondria. Incubation mixture consisted of 0.1 M sucrose, 20 mM Tris-HCl buffer (pH 7.4), 40 μM EDTA, 10 mM $MgCl_2$, 3 mM Na-succinate, 3 mM ATP, 3 mM K-phosphate buffer (pH 7.4 containing 5 μc of P^{32}) and 5 mg protein of mitochondria in 3 ml and incubated at 25° for indicated time. Other additions described in the table.

Incubation system addition (+) or omission (−)	Incubation time (min.)	Counts of P^{32} accumulated in mitochondria (c.p.m.)
Complete	3	7834
〃	10	9357
− mitochondria	3	969
− Ca^{2+}	3	1460
− ATP	3	1628
− Mg^{2+}	3	1407
− succinate	3	2199
+ DNP (0.03 mM)	3	3953
+ antimycin A (2 γ/ml)	3	2446
+ oligomycin (0.3 γ/ml)	3	10483
+ TBTC (0.2 μM)	3	10066
+ oligomycin + antimycin A	3	1920
+ TBTC + antimycin A	3	1982

Table 2. Accumulation of Ca^{45} in rat liver mitochondria. Incubation mixture consisted of 0.1 M sucrose, 10 mM Tris-HCl buffer (pH 7.4), 20 mM KCl, 3 mM Na-succinate, 3 mM ATP, 3 mM K-Phosphate buffer (pH 7.4), 1 mM $CaCl_2$ (containing 30 μc of Ca^{45}) and 3.42 mg protein of mitochondria. Incubation carried out at 25° for indicated time. Other additions described in the table.

Incubation system addition (+) or omission (−)	Incubation time (min.)	Counts of Ca^{45} accumulated in mitochondria (c.p.m.)
Complete	3	26243
〃	10	41989
〃	20	62985
− mitochondria	3	295
− Pi	3	1362
− ATP	3	1247
− Mg^{2+}	3	845
− succinate	3	4439
+ DNP (0.03 mM)	3	7173
+ antimycin A (2 γ/ml)	3	9948
+ oligomycin (0.3 γ/ml)	3	31299
+ TBTC (0.2 μM)	3	28904
+ antimycin A + oligomycin	3	3454
+ antimycin + TBTC	3	1617

the accumtlation of P^{32} in the mitochondrial Pi fraction in high concentration of $MgCl_2$ (10 mM) and $CaCl_2$ (1 mM) at 25°. The amount of accumulated P^{32} was increased in proportion to the time of incubation. This accumulation required Ca^{2+}, ATP, Mg^{2+} and succinate, and was inhibited by respiratory inhibitors (KCN and antimycin A) and uncouplers of oxidative phosphorylation (DNP, oleic acid and dicumarol) but was not affected or was stimulated slightly by inhibitors of phosphorylating respiration (oligomycin and TBTC). The addition of oligomycin to the medium containing antimycin A resulted in an extreme decrease in the Pi accumulation. The accumulation of Ca^{45} in the mitochondria was also observed in a medium similar to that used for pursuing P^{32} incorporation at 25°. This Ca^{45} uptake required Pi, ATP, Mg^{2+} and succinate and the inhibitor pattern was quite similar to that of P^{32} inconporation. In this instance, however, the inhibition of Ca^{45} accumulation by antimycin was weak and it was promoted by addition of oligomycin or TBTC as shown in Table 2.

Accumulation of Ca^{45} in rat liver mitochondria in the medium containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$: The data presented in Table 3 show the effects of number of metabolic inhibitors and the requirement for Ca^{45} accumulation in the presence of 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ at 25°. This Ca^{2+} accumulation did not require Mg^{2+} and was inhibited slightly by ATP. But

Table 3. Translocation of Ca^{45} in rat liver mitochondria. Incubation mixture consisted of 0.1 M sucrose, 10 mM Tris-HCl buffer (bH 7.4), 1 mM $MgCl_2$, 3 mM K-phosphate buffer (pH 7.4), 3 mM Na-succinate, 20 mM KCl, 0.1 mM $CaCl_2$ (containing 10 μc of Ca^{45}) and 2.6 mg protein of mitochondria. Incubation carried out at 25° for indicated time. Other additions described in the table.

Incubation system addition (+) or omission (−)	Incubation time (min.)	Counts of Ca^{45} translocated in mitochondria (c.p.m.)
Complete	3	3401
〃	10	2810
〃	50	297
− mitochondria	3	235
− Mg^{2+}	3	3701
− succinate	3	322
+ DNP (0.1 mM)	3	396
+ antimycin A (2 γ/ml)	3	238
+ oligomycin (0.3 γ/ml)	3	4111
+ TBTC (0.2 μM)	3	4016
+ antimycin + oligomycin	3	237
+ ATP (3 mM)	3	1432
+ ATP + antimycin A	3	1131
+ ATP + oligomycin	3	2958
+ ATP + antimycin A + oligomycin	3	834

the amount of Ca^{45} accumulated in mitochondria was smaller in comparison with that observed in the presence of 1 mM $CaCl_2$, 3 mM ATP, 10 mM $MgCl_2$ and 3 mM succinate. The inhibitor pattern of Ca^{45} by metabolic inhibitors was similar to that in high concentration of $CaCl_2$ (1 mM), but the Ca^{45} accumulation could still be observed by addition of ATP even in the presence of antimycin A, which is sensitive to oligomycin. The pattern of P^{32} accumulation under the same conditions was similar to that of Ca^{45} accumulation, although the measurement was quite difficult.

Translocation of P^{32} and Ca^{45} in mitochondria at a low temperature: A persistency in structural change induced experimentally in mitochondria often brings about several difficulties. For instance, it is difficult to measure the amount of Ca^{45} taken up by mitochondria because in the presence of Pi a remarkable swelling of mitochondria was induced by addition of Ca^{2+} as shown in Fig. 1. Consequently, the incubation was carried out at a low temperature to prevent the mitochondrial swelling. Table 4 shows a rapid incorporation of P^{32} into mitochondrial Pi fraction at low temperature (in ice cold) in a medium similar to that used in the former experiment containing 0.1 mM $CaCl_2$, 1 mM

Table 4. Translocation of P^{32} in rat liver mitochondria at a low temperature. Incubation mixture consisted of 0.1 M sucrose, 50 mM Tris-HCl buffer (pH 7.4), 20 mM KCl, 3 mM K-phosphate buffer (pH 7.4 containing 20 μc of P^{32}), 1 mM $MgCl_2$, 40 μM EDTA, 0.1 mM $CaCl_2$ and 3.1 mg protein of mitochondria. Incubation carried out in ice cold for indicated time. Other additions described in the table.

Incubation system addition (+) or omission (−)	Incubation time (min.)	Pi translocated. μmole/mg	Counts of P^{32} translocated in mitochondira (c.p.m)
Complete	0	400	4440
〃	3	545	5639
〃	5	585	6539
〃	10	631	6508
− mitochondria	3	—	1317
− Ca^{2+}	3	140	1523
− Mg^{2+}	3	400	5538
+ ATP (3 mM)	3	382	2765
+ ADP (1 mM)	3	344	2503
+ DNP (0.03 mM)	3	56	1392
+ antimycin A (2 γ/ml)	3	74	1590
+ oligomycin (1.8 γ/ml)	3	535	5023
+ succinate (3 mM)	3	580	6174
+ ATP + antimycin A	3	91	1832
+ ATP + antimycin A + oligomycin	3	83	1625

$MgCl_2$ and no ATP. This P^{32} uptake was brought about in the absence of exogenous respiratory substrate and Mg^{2+}, but requiring Ca^{2+}, and was accelerated slightly by succinate. Addition of ATP to the complete medium appeared to reduce rather than to enhance the P^{32} translocation. The inhibitor pattern was similar to that observed at 25°. The Ca^{45} translocation proceeded at low temperature, as reveled in Table 5, and the requirement and inhibibtor pattern were

Translocation of Ca^{45} in rat liver mitochondria at a low temperature. Incubation mixture consisted of 0.1 M sucrose, 10 mM Tris-HCl buffer (pH 7.4), 20 mM KCl, 3 mM K-phosphate buffer (pH 7.4), 1 mM $MgCl_2$, 0.1 mM $CaCl_2$ (containing 20 μc of Ca^{45}) and 3 mM Na-succinate or α-KG. The mitochondrial protein was 3.54, 3.4 and 3.5 for endogenous, α-KG and succinate respectively. Incubation carried out in ice cold for indicated time. Other additions described in the table.

Incubation system addition (+) or omission (−)	Incubation time (min.)	Total counts of Ca^{45} translocated im mitochondria (c.p.m.)		
		endogenous	α-KG	succinate
Complete	0	1817	1617	2468
〃	1	2557	3761	4252
〃	3	4418	5107	5544
〃	10	4605	5249	6651
〃	20	4282	4983	6334
− mitochondria	3	178	165	235
− substrate	3	—	4250	3820
− Mg^{2+}	3	4692	4392	5897
− Pi	3	1725	—	—
+ DNP (0.1 mM)	3	593	860	792
+ antimycin A (2 γ/ml)	3	542	755	876
+ pligomycin (2 γ/ml)	3	5102	4513	5222
+ ATP (3 mM)	3	3100	—	3762
+ ATP + antimycin A	3	1545	1264	2025
+ ATP + oligomycin	3	4782	—	—
+ ATP + antimycin A + oligomycin	3	820	553	1142

quite similar to those of the P^{32} translocation at low temperature, in the medium containing succinate or pyridine nucleotide linked substrate (α-KG or glutamate) and/or no exogenous substrate. The increase of the translocation was frequently observed by addition of oligomycin at a relatively low concentration than at a higher concentration.

DISCUSSION

The present and previous[8] findings demonstrate that the close correlationship exists between the mitochondrial swelling and ion accumulation (Pi and Ca^{2+}).

$CaCl_2$ rapidly reduces the turbidity of the mitochondrial suspension by 15—40 per cent in the presence of Pi as revealed in the present experiment, but the concentration of $CaCl_2$ is insufficient to induce high amplitude swelling: this swelling was much less extensive than other fragmenting treatment[5,20,21], but greater than reversible swelling and shrinkage observed on addition of substrate, Pi and ADP[8,9,22] as described PUMPHREY and REDFEARN[14].

Since the initial rapid shrinkage by addition of Ca^{2+} coincides with the temporary stimulation of oxygen uptake and with rapid oxidation of pyridine nucleotides in the presence or absence of exogenous substrate, it is presumed that these initial rapid changes are relevant to those changes observable in enzyme activity. This idea is supported by the experimental results that the increased shrinkage and oxidation of pyridine nucleotides by Ca^{2+} are observed at the same time in the presence of succinate, and after the brake of the increased respiration, re-reduction of pyridine nucleotides occurs being accompanied by the swelling of mitochondria. Therefore, the shrinkage induced by a small amount of Ca^{2+} (0.1 mM) in the presence of Pi corresponds to the low amplituded swelling of PACKER[9]. The mechanism of this shrinkage may be explained as follows: this shrinkage is coupled with the oxidative phosphorylation because it does not occur on the addition of DNP or dicumarol. Moreover, since Ca^{2+} at a low concetration do partially uncouple the oxidative phosphorylation and increases the ADP concentration in mitochondria by Ca^{2+} induced ATPase activity, it is presumable that the respiratory control by ADP[23] appears in accompaniment with the shrinkage of mitochondria as described in previous paper[8,22]. In this case the brake of increased respiration and the re-reduction of pyridine nucleotides are due to the initial burst of ATPase activity by Ca^{2+}[24].

Pyridine nucleotides are oxidized by a small amount of Ca^{2+} with or without Pi suggesting the translocation of Ca^{2+} into the mitochondria without Pi. This translocation of Ca^{2+} agrees with the experimental result of CHAPPELL *et al*[25] which shows the active transport of divalent cations without Pi, but phosphate is required for rapid uncoupling of respiration and for the swelling by Ca^{2+}. This Pi-dependent Ca^{2+} induced swelling is inhibited by respiratory inhibitors and uncouplers of oxidative phosphorylatnon and not by inhibitors of phosphorylating respiration. Moreover, it is important to emphasize that the Ca^{2+} induced swelling is elicited by ATP even in the presence of antimycin A, which is sensitive to oligomycin. Therefore, these findings suggest that the Ca^{2+} induced swelling is an energy dependent reaction and that the energy comes apparently from the oxidative phosphorylation process[26,27]. In this instance, the fact that the Pi-dependent Ca^{2+} induced swelling can occur in oligomycin-blocked mitochondria suggests that an intermediate of oxidative phosphorylation before the oligomycin block may provide the energy for the Ca^{2+} induced swelling.

The degree of Pi-dependent Ca^{2+} induced swelling is decreased in proportion to the decrease of respiratory activity of mitochondria in the following order of endogenous, pyridine nucleotide linked substrate and succinate. The inhibition of the Ca^{2+} induced swelling is observed by the addition of ATP. The relationship of this and the requirement of intermediate for Ca^{2+} induced swelling must await further experimental examinations. When mitochondria are incubated with succinate or ATP, the degree of swelling induced by Ca^{2+} is reduced, so that it is presumed that the swelling is controlled by intramitochondrial ATP content and the amount of translocated Ca^{2+}.

As described by many investigators the swelling of mitochondria is associated with change in intramitochondrial water and ions[28,29]. The requirement and inhibitor pattern of Ca^{2+} induced swelling and of Ca^{2+} accumulation in a small amount are quite similar as estimated in this paper, but the requirements of ATP, Mg^{2+} and substrate are different between the Ca^{2+} induced swelling and Ca^{2+} accumulation in large amounts. In this instance, it is difficult to measure the amount of Ca^{2+} taken up by mitochondria in swollen state as indicated in Fig. 1. Thus it is considered that the difference between the swelling by Ca^{2+} and the Ca^{2+} uptake is the result of swollen state of mitochondria. In fact, when mitochondria are incubated at a low temperature with Ca^{2+}, Ca^{2+} accumulation proceeds without the Mg^{2+}, ATP and exogenous respiratory substrates. Therefore, the mitochondrial swelling induced by Ca^{2+} proceeds in parallel with the Ca^{2+} accumulation which is in a small amount and proceeds proportional to the time of incubation under restored structural change of mitochondria in low amplitude swelling. This ion accumulation is also inhibited by respiratory inhibitors or uncouplers of oxidative phosphorylation but is accelerated frequently by oligomycin. The increased accumulation of ion by oligomycin can be explained by the increment of an intermediate, an initial step of oxidative phosphorylation sequence which can accelerate the ion accumulation by its high energy, in oligomycin blocked mitochondria. Thus the Ca^{2+} induced swelling is closely correlated to the Ca^{2+} accumulation in relation to the intermediate of initial step of oxidative phosphorylation processes. Pi accumulation at low temperature, on the other hand, is brought about in the medium similar to that used for Ca^{2+} accumulation, and the requirement and inhibitor pattern are similar to those observed in the case of Ca^{2+} accumulation. Concerning the Mg^{2+}, Ca^{2+}, Mn^{2+} and Pi accumulation, several investigators[30,31,25] claim that the Ca^{2+} or Mg^{2+} and/or Mn^{2+} enter in a definite molar ratio and the stoichiometric relationships exist between the amounts of Ca^{2+} and Pi accumulated by respirating mitochondria[2]. The results of the present experiment also are not an exception but Ca^{2+} are transported into mitochondria even in the absence of Pi as indicated by CHAPPELL *et al*[25]. As illustrated in previous paper[8], although, the author de-

scribed that Pi accumulation proceeded in the medium having no exogenous Ca^{2+} or Mg^{2+} but there still remain a question whether the Pi accumulation is due to the active transport without Ca^{2+} or dependent on the endogenous Ca^{2+}. On this problem, recently CHAPPELL *et al.*[25] represented a scheme for Mn^{2+} accumulation and described the passive transport of Pi and active transport of Mn^{2+}. LEHNINGER[33] described that the ratio atom Ca^{2+} accumnlated : atoms oxygen utilized was found to be of the same order of magnitude as the moral P : O ratio of oxidative phosphorylation. The transport of Ca^{2+} and Pi at low temperature shows its dependency on the high energy intermediate as can be understood from the fact that the inhibition of these ion accumulation by DNP or oleic acid can be observed. The mechanism operating here with respect to the electron transport is obscure because the transport is inhibited by antimycin A but proceeds even in the incubation at a low temperature without oxygen uptake.

In any case, the active transport of Ca^{2+} is brought about along with its uncouping action. A convincing interpretation of the uncoupling action of Ca^{2+} is that it is produced directly by accepting the energy for transport or binding to certain mitochondrial substance. In this case phosphate is required for a rapid uncoupling of respiration, for the swelling by $Ca^{2+}$and for the accumulation of Ca^{2+}. This means that a phosphorylated intermediate in the energy transfer sequence plays a part in Ca^{2+} accumulation and the uncoupling and swelling by Ca^{2+}, or that Pi induces the accumulation of a large amount of Ca^{2+} using high energy intermediate by forming the calcium phosphate, and then uncoupling is brought about to be accompanied with the alteration of mitochondrial structure.

Since Pi is required for the swelling of mitochondria induced by succinate or oleic acid in the medium containing EDTA, which is sensitive to antimycin A and DNP but not to oligomycin suggesting its dependency on oxidative phosphorylation, it is considered that the site of energy transducing sequence for the low amplitude swelling or shrinkage is an intermediate step before oligomycin block. In addition to this, the step has some interaction to phosphate or phosphate compound. For the solution of this problem further experiments need be carried out.

SUMMARY

Some investigations have been done on the relationships between the swelling-shrinkage change, oxygen consumption and state of oxidation-reduction of pyridine nucleotides of mitochondria, and between the swelling-shrinkage change of mitochondrial structure by Ca^{2+} and accumlation of Ca^{45} in rat liver mitochondria. A parallel relationship is observed between the Ca^{2+} induced swelling and Ca^{2+} accumulation. Both of them require Pi but not Mg^{2+}, ATP and exogenous respiratory substrates and are inhibited by respiratory inhihitors or uncouplers of

oxidative phosphorylation but not by the inhibitors of phosphorylating respiration. In this case the Ca^{2+} is transported with the phosphate even in ice cold. Even in the presence of antimycin A, moreover, Pi-dependent Ca^{2+} accumulation and Ca^{2+} induced swelling can be overcome by addition of ATP, which are inhibited by oligomycin.

In the presence of Pi, mitochondria show shrinkage by addition of Ca^{2+} before the high amplitude swelling, which is closely correlated to the electron transport chain and phosphorylation process of mitochondria, and the pattern of the mitochondrial shrinkage is quite similar to that observed in the case of respiratory control by ADP in intact mitochondria. This shrinkage of mitochondria is inhibited by respiratory inhibitor or uncoupler of oxidative phosphorylation but not by the inhibitor of phosphorylating respiration.

From these data, therefore, it is considerd that the Ca^{2+} accumulation and Ca^{2+} induced shrinkage-swelling of mitochondria require the energy of oxidative phosphorylation with respect to the initial step before the oligomycin block.

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