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Cellular Organelles

Energy-Independent Protection of the Oxidative Phosphorylation Capacity of Mitochondria against Anoxic Damage by ATP and Its Non-Metabolizable Analogs.

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Running title: Protection of Oxidative Phosphorylation Capacity

by ATP

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Abbreviations: AMPPCP, β , γ -methylene adenosine 5'-triphosphate; AMPCPP, α , β -methylene adenosine 5'-triphosphate; AMPPNP, adenylyl imidodiphosphate; AMPCP, α , β -methylene adenosine 5'diphosphate; FCCP, carbonyl cyanide <u>p</u>-trifluoromethoxyphenyl hydrazone.

SUMMARY

Preservation of the oxidative phosphorylation capacity of mitochondria by addition of ATP under anaerobic conditions was analyzed by use of non-metabolizable adenine nucleotide analogs. The capacity was well preserved in the presence of ATP, and did not require the hydrolysis of ATP, since ATP analogs, such as β, r -methylene adenosine triphosphate (AMPPCP), β, β -methylene adenosine triphosphate (AMPCPP), and adenylyl imidodiphosphate (AMPPNP), were as effective as ATP. These analogs were incorporated into mitochondria through ATP/ADP translocase to maintain the original level of total adenine nucleotides in the mitochondria. ADP apparently had the same effect as ATP, but its effect was shown to be due to ATP generated from it by adenylatekinase in mitochondria. An analog of ADP, α,β -methylene adenosine diphosphate (AMPCP), which was found to be a substrate of the translocase but not of adenylatekinase, could not replace ADP or ATP. From these results, it was concluded that the oxidative phosphorylation capacity of mitochondria was maintained by ATP, but not ADP, through a process not requiring energy.

Prolonged interruption of the blood supply to an organ injures the cells, leading to their death. There are reports that the irreversible damage caused by ischemia is due to loss of the oxidative phosphorylation capacity of mitochondria (1-5). Several mechanisms have been proposed for this dysfunction of mitochondria in ischemic tissue, such as impairment of the electron transfer system caused by denaturation of cytochromes (4) and inhibition of ATP/ADP translocase by long chain acyl CoA accumulating during ischemia (2,4). Previously, we reported that mitochondria in ischemic rat liver lost the capacity for oxidative phosphorylation with concomitant decrease in the intramitochondrial level of ATP plus ADP, and that this resulted in apparent inactivation of ATP/ADP translocase because of decrease in the intramitochondrial substrate to antiport externally added ADP or ATP (6). We also showed that the phosphorylation capacity of isolated mitochondria was preserved when the intramitochondrial levels of ATP and ADP were maintained either by suppressing hydrolysis of the nucleotides by incubation at low temperature, or by adding ATP to the external medium, or by allowing mitochondria to respire by adding a respiratory substrate under aerobic conditions. It is readily understandable that mitochondria require certain internal levels of ADP and ATP for oxidative phosphorylation because ATP inside mitochondria is essential for import of external ADP via ATP/ADP translocase and ADP is the sole phosphate acceptor in oxidative phosphorylation.

However, several workers have postulated that besides being a direct substrate for essential enzymes, ATP is required for stabilizing mitochondria (7), for synthesis of co-factors, such

as FAD ($\underline{8}$), and as an activator of the electron transfer system ($\underline{3}$). Romani and his co-workers reported that the capacity for oxidative phosphorylation is maintained by energy that is supplied essentially by respiration ($\underline{9-12}$). In the present investigation, several kinds of non-metabolizable analogs of ATP were found to be as effective as ATP itself in preservating the oxidative phosphorylation capacity of isolated mitochondria, whereas an analog of ADP, which could be a good substrate of ATP/ADP translocase and maintain the enzyme activity as well as the original level of nucleotides inside mitochondria, did not protect mitochondria against anoxic damage. Thus, we concluded that ATP is required inside mitochondria to maintain their oxidative phosphorylation capacity <u>in vivo</u>, but that for its effect the nucleotide is not used as an energy source.

MATERIALS AND METHODS

<u>Preparation of Mitochondria</u> ----- Male Sprague-Dawley rats weighing about 300-400 g were used. They were starved overnight and then anesthetized by intraperitoneal injection of ketamine at 100 mg/kg body weight. Mitochondria were isolated from their liver in 0.3 M mannitol containing 0.1 mM EDTA, pH 7.4, and 0.1 % bovine serum albumin (6). Mitochondrial protein was determined by the method of Lowry et al. (13).

Incubation of Mitochondria and Their Separation from the Incubation Medium ----- Mitochondria, 10-17 mg protein/ml, isolated from normal rat liver were incubated with various species of nucleotides under anoxic conditions at 25° C. The incubation medium consisted of 0.3 M mannitol, 10 mM Tris-HCl, pH 7.4, 5 mM potassium phosphate, 0.2 mM EDTA, 0.1 % bovine serum albumin, and 1 mM MgCl₂. After incubation, the mitochondria were separated from the medium by the centrifuge column method described previously (<u>6,14</u>). The amount of adenine nucleotides and the respiration rate of the mitochondria were measured after their separation, since this separation procedure did not affect the respiratory activity of the mitochondria.

Evaluation of Oxidative Phosphorylation Capacity of <u>Mitochondria</u> ---- Oxidative phosphorylation capacity was evaluated with the respiratory control ratio which has been used as a sensitive and conventional indicator of the capacity (4, 6). The rate of oxygen consumption by isolated mitochondria was measured with a Clark-type electrode at 25°C in incubation medium with succinate as a substrate.

Measurement of Adenine Nucleotides and Their Analogs in

Mitochondria ---- Adenine nucleotides and their analogs in mitochondria were extracted with 0.5 N perchloric acid and the extract was neutralized with 10 N potassium hydroxide. The nucleotides were determined by high performance liquid chromatography on an anion exchange column (4.6 x 250 mm) of DEAE-2SW equilibrated with 360 mM sodium phosphate buffer, pH 6.0, at a flow rate of 1.0 ml/min (6,14,15,16). All species used in this study were well separated from each other with high theoretical plate numbers (about 5400) and could be measured with a precision of about 10 pmol within 17 min. The retention times of ATP, ADP, AMP, AMPCP, AMPPNP, AMPCPP, and AMPPCP were 16.5, 10.4, 7.1, 7.6, 12.4, 12.1, and 11.9 min, respectively. For separation of the last two analogs, the column was developed with buffer containing 200 mM sodium phosphate, pH 2.8, which eluted AMPCP, ADP, AMPPCP, and AMPCPP at 5.5, 7.7, 13.4, and 15.2 min, respectively.

RESULTS

<u>Stability of ATP Analogs in Mitochondrial Suspension</u> ---- As reported previously, ATP is slowly hydrolyzed when added to a mitochondrial suspension during anoxic incubation (<u>6</u>). The metabolic stabilities of the ATP analogs used in the present study were examined by comparing their rates of hydrolysis with that of ATP. As shown in Fig.1, AMPCPP and AMPPNP were fairly stable during the first 60 min of incubation, but then were slowly hydrolyzed to the corresponding dinucleotides, AMPCP and

Fig. 1

ADP, respectively. On the other hand, AMPPCP was very stable and was not hydrolyzed within 180 min. Therefore, this last analog was mainly used in following experiments to determine whether hydrolysis of ATP is essential for protection of the oxidative phosphorylation capacity of mitochondria.

<u>Preservation of the Oxidative Phosphorylation Capacity of</u> <u>Mitochondria by Non-metabolizable Analogs of ATP</u> ---- In the absence of ATP, the oxidative phosphorylation capacity of isolated mitochondria was lost within 120 min during anoxic incubation, as judged from the decrease in the respiratory control ratio (Fig.2A). The decrease was partly due to decrease in the rate of state 3 respiration but mainly to increase in that

Fig.2 A,B

of state 4 respiration. The increase of state 4 respiration rate was not attributable to activation of F_{O1} -ATPase, but to impairment of proton tightness of the inner membrane, since it

was not suppressed by oligomycin. Actually, the permeability of H⁺ across the inner membrane of mitochondria incubated for 3 hr was 27 times as larger as that of intact mitochondria (Watanabe, F., unpublished data). The electron transfer system of mitochondria was unimpaired because the respiration rate released by carbonyl cyanide p-trifluoromethoxyphenyl hydrazone (FCCP) remained constant during the incubation. As reported previously (6), the oxidative phosphorylation capacity of the mitochondria was protected against anoxic damage by the addition of ATP. In the present study, we found that for this protective effect, ATP could be replaced by AMPPCP, which was not hydrolyzed during incubation. Figure 2B shows that the ATP analog effectively prevented increase in state 4 respiration. The two other analogs AMPCPP and AMPPNP also had similar effects to ATP. Table I summarizes the effects of these analogs on changes in the respiratory control ratio during anoxic incubation, in comparison

Table I

with that of ATP itself. Although there was a slight decrease in the respiratory control ratio after 2hr incubation, the results clearly show that these analogs all had protective effects, and therefore that no supply of metabolic energy was required to protect mitochondrial oxidative phosphorylation.

<u>Incorporation of Analogs of ATP into Mitochondria</u> ---- As reported previously, mitochondria capable of phosphorylation maintain a definite internal level of ATP+ADP (<u>6</u>). Analogs of ATP added to maintain phosphorylation capacity were also found to be incorporated into mitochondria and to replace ATP and ADP.

Figure 3 shows that added AMPPCP was rapidly exchanged with intramitochondrial ATP and was maintained at a high level during anoxic incubation. This incorporated analog was also quickly exchanged with externally added ADP, causing no inhibition of

Fig. 3

state 3 respiration. The exchange of the analog with ATP or ADP was probably mediated by ATP/ADP translocase, since the exchange was strongly inhibited by addition of atractyloside (Fig.4A). In the presence of the inhibitor, more than 99% of the enzyme activity was inhibited but AMPPCP was gradually incorporated into

Fig. 4 A,B

mitochondria by the residual activity, reaching a maximum level in about 120 min. This slowly incorporated analog was also effective in protecting the oxidative phosphorylation capacity (Fig.4B). In this experiment, state 3 respiration could not be measured accurately because of the presence of atractyloside, but the respiratory control ratio was determined from the respiration rate released by FCCP and the rate of state 4 respiration.

<u>Absence of Protective Effect of ADP Analog</u> ---- Added ADP also protected the oxidative phosphorylation capacity. Its protective effect was, however, attributable to that of ATP formed from ADP by mitochondrial adenylatekinase. More than half the total of ADP added to the mitochondrial suspension was converted to ATP and AMP within a minute. The protective action of ADP was not abolished in the presence of diadenosine pentaphosphate, an inhibitor of muscle adenylatekinase, but it

was found that this compound did not inhibit the conversion

Table II

occurring in mitochondria (Table II). Moreover, at concentrations of below 1 mM ADP was much less effective than ATP in protecting the oxidative phosphorylation capacity (data not shown). The absence of a direct action of ADP was more clearly shown by use of the ADP analog AMPCP instead of ADP. The analog was rapidly exchanged with intramitochondrial ATP and ADP and a small portion of the analog was converted to its triphosphate analog, AMPCPP (Fig.5A). The exchange with natural nucleotide was so efficient that mitochondrial adenine nucleotides could be exclusively replaced by the analog and its phosphorylated product

Fig. 5 A,B

and the nucleotide level in mitochondria was maintained during incubation. However, state 4 respiration increased as observed in the absence of ATP (Fig.5B and Fig.2A). It was noticed, however, that during this deterioration process there was no dècrease of state 3 respiration, in contrast to the case without added nucleotide. This suggests that the translocase was unimpaired and that intramitochondrial substrates were available for the enzyme. Actually, AMPCP incorporated into mitochondria was found to be readily exchanged with AMPPCP even after 180 min incubation, when the respiratoy control had been completely lost (Table III).

Table III

DISCUSSION

It has long been known that externally added ATP and ADP protect the oxidative phosphorylation capacity of mitochondrial suspension against anoxic damage (3,7,8,17). The protective action has been explained by supposing that the nucleotides provide an energy supply for the oxidative phosphorylation system (17). In the present study, however, we showed that for its protective action ATP could be replaced by non-metabolizable analogs, such as AMPPCP, AMPCPP and AMPPNP. These analogs were incorporated into mitochondria by antiport with natural nucleotides inside the mitochondria and that all the analogs protected the oxidative phosphorylation capacity against anoxic damage. Thus it may be concluded that preservation of the oxidative phosphorylation capacity of mitochondria requires the presence of ATP inside the mitochondria but nor the hydrolysis of ATP or energy supply from ATP. This conclusion rules out the direct protective effect of ADP, and consistent with this, the non-metabolizable analog of ADP, AMPCP, did not have any protective effect.

Some reports are inconsistent with our conclusion. For instance, Romani <u>et al.</u> reported that for protection, energy supplied by respiration was effective but ATP was not (9-12). However, as reported previously, addition of a respiratory substrate under anaerobic conditions maintains the level of total adenine nucleotide in mitochondria, and therefore its protective action can be attributed to its effect on the intramitochondrial level of ATP (<u>6</u>). Another inconsistent report was that ATP is required for the electron transfer system as an activator, since

a close correlation was observed between decrease in the rate of state 3 respiration and decrease in the ATP level in mitochondria isolated from hypoxic perfused rat liver ($\underline{3}$). In this experiment, however, respiration released in the presence of uncoupler should have been measured and taken as for the maximum respiration rate instead of state 3 respiration, because as shown in Fig.2A, state 3 respiration apparently decreased during anoxic incubation while FCCP-released respiration did not change. Our observation is consistent with that of Asimakis and Aprille ($\underline{18}$) that when the level of adenine nucleotides in mitochondria becomes low, ATP/ADP translocase becomes rate-limiting for state 3 respiration.

It is unknown why ATP is required to protect mitochondrial function. However, the fact that the inner membrane becomes permeable to H^+ in the absence of ATP strongly suggests the existence of some membrane protein that is integrated into the inner membrane properly only when bound to ATP. Another possibility is that there is a ligand gated-channel protein that is specific for H^+ , and that closes on binding to ATP. The latter possibility is analogous to the uncoupling mechanism elucidated recently in mitochondria of brown adipose tissue $(\underline{19, 20})$.

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Table I. Maintenance of oxidative phosphorylation capacity of mitochondria by addition of ATP analogs. Data were obtained in the same experiments as for Fig.1 and Fig.2A.

ncubation time	Respiratory control ratio				
(min)	Control	ATP	AMPPCP	AMPCPP	AMPPNE
0	7.8	7.9	7.8	7.1	6.9
60	3.2	4.9	4.5	3.7	5.9
120	1.0	4.8	3.6	3.1	2.8

Table II. Conversion of ADP to ATP and AMP by the action of adenylatekinase in mitochondria. Mitochondria, 9.1 mg protein/ ml, were incubated in incubation medium in the presence of 0.1 mM diadenosine pentaphosphate at 25°C. ADP at a final concentration of 5.6 mM was added to the suspension. At the times indicated, samples of 200 jul were removed for measurement of adenine nucleotides in the medium.

	AMP	ADP	ATP (mM)
0 min 5 min	0.2	5.3	0.1 2.1

Table III. Exchange of AMPCP preloaded in mitochondria with AMPPCP added externally. Mitochondria, 17 mg protein/ml, were incubated with 5 mM AMPCP for 180 min in the incubation medium and then separated from the medium by the centrifuge column method. Before (A) and after (B) incubation, the contents of adenine nucleotides and analogs in the mitochondria were assayed. Then, the mitochondrial suspension separated from the first incubation medium, were incubated with AMPPCP at a final concentration of 5 mM for 5 min. Adenine nucleotides and the incorporated analogs in the mitochondria were assayed after the second centrifuge column treatment (C).

Contents in mitochondria (nmol/mg protein)

		AMP+ADP+ATP	AMPCP+AMPCPP	АМРРСР		
(A)	Preincubation	22.2	0.0	0.0		
(B)	Incubation with AMPCP 180 min	0.0	18.3	0.0		
(C)	(B) + AMPPCP	2.3	2.1	12.1		

Fig.1. Stability of AMPPCP incubated in mitochondrial suspension. ATP and its analogs were incubated with 14 mg protein/ml of mitochondrial suspension at 25°C. Adenine nucleotides and analogs were extracted with 0.5N perchloric acid after the incubation and analyzed by high performance liquid chromatography.

(O), ATP; (\triangle), AMPPCP; (\bigcirc), AMPPNP; (\Box), AMPCPP

Fig.2. Maintenance of oxidative phosphorylation capacity of mitochondria by AMPPCP. Mitochondria, 14 mg protein/ml, were incubated at 25° C in the presence of 5.6 mM of AMPPCP (B) and in its absence (A). At the times indicated, mitochondria were separated from the medium by the centrifuge column methods and their respiration rate was measured. (O), state 3 respiration rate; (\bullet), state 4 respiration rate; (\bullet), respiration rate in the presence of oligomycin; (\blacktriangle), respiration rate in the presence of form the presence of

Fig.3. Retention of adenine nucleotide levels in mitochondria incubated with AMPPCP. Data were obtained in the same experiment as for Fig.2A. (○), ATP; (□), ADP; (▲), AMP; (Ⅲ), AMPPCP; (●), AMP+ADP+ATP+AMPPCP

Fig.4. Slow incorporation of AMPPCP into mitochondria and protection of oxidative phosphorylation capacity. Mitochondria, 14 mg protein/ml, were incubated at 25 °C in the presence of 50 μ M atractyloside and 5.6 mM AMPPCP. At the times indicated, mitochondria were separated from the medium by the centrifuge column method and their adenine nucleotide contents (A) and respiration rate (B) were measured. (A), (O), ATP; (\Box), ADP; (\blacktriangle), AMP; (\blacksquare), AMPPCP; (\bullet), ATP+ADP+AMP+AMPPCP (B), (\bullet), state 4 respiration; (\bigstar), respiration in the presence of FCCP.

Fig.5. Changes in levels of adenine nucleotides and respiration rate of mitochondria incubated with ADP analog. Mitochondria, 9.4 mg protein/ml, were incubated in medium containing 5.2 mM AMPCP. At the times indicated, mitochondria were separated from the medium and their adenine nucleotide levels (A) and respiration activity (B) were measured. (A) (\blacktriangle), AMPCP; (\square), AMPCPP; (\bullet), AMP+ADP+ATP+AMPCPP+AMPCP (B) (O), state 3 respiration rate; (\bullet), state 4 respiration rate; (\blacksquare), respiration rate in the presence of oligomycin; (\bigstar), respiration rate in the presence of FCCP.



Fi.g. 1



Fig. 2





Fig.4



Fig. 5



Decrease in Mitochondrial Levels of Adenine Nucleotides and Concomitant Mitochondrial Dysfunction in Ischemic Rat Liver¹

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Decrease in Mitochondrial Levels of Adenine Nucleotides and Concomitant Mitochondrial Dysfunction in Ischemic Rat Liver¹

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The process of mitochondrial dysfunction in ischemic rat liver was studied. A close correlation was found between decrease in the mitochondrial adenine nucleotide content and deterioration of oxidative phosphorylation capacity. The level of total adenine nucleotides, which was 15-20 nmol/mg protein in mitochondria isolated from normal liver, fell to 1-2 nmol/mg protein with concomitant loss of oxidative phosphorylation capacity after anoxic incubation in vitro or in vivo for 120 min. However, neither the permeability barrier to adenine nucleotides nor matrix enzymes were affected under these conditions. The loss of adenine nucleotides was ascribed to degradation of AMP to adenosine and then leakage of the latter. Conventional procedures for maintenance of oxidative phosphorylation capacity of isolated mitochondria, preservation in the cold and addition of ATP or a respiratory substrate under aerobic conditions, were very effective in maintaining the intramitochondrial levels of adenine nucleotides. Of the three species of adenine nucleotides, only AMP was ineffective in maintaining mitochondrial function; mitochondria containing more than 5 nmol of ATP plus ADP/mg protein exhibited normal activity of oxidative phosphorylation, but with less than 2 nmol they showed no activity.

When prolonged, ischemia causes irreversible injury of liver cells, and finally cell death. This irreversible cell injury during prolonged ischemia was reported to be caused by loss of oxidative phosphorylation capacity of mitochondria (1-5). Various events have been proposed to explain ischemic mitochondrial dysfunction, such as losses of mitochondrial components, *e.g.* phospholipids (6) and cytochromes (5), and inhibition of ATP/ ADP translocase by long chain acyl-CoA, which increases during ischemia (3-5). However, despite many studies on this problem, it is premature to draw any definite conclusions from changes in constituents about the sequence of events in ischemic mitochondrial dysfunction.

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Abbreviation: F_0F_1 -ATPase, oligomycin-sensitive ATPase in mitochondria.

Previously, we reported that the cellular level of ATP in the liver became almost zero during prolonged ischemia, and was not restored during recirculation (7). The fact that the level of ATP in ischemic liver could not be restored may merely be due to depletion of available ATP, since a small amount of ATP may be necessary for the initial step of its own production. Actually, Nakazawa and Nunokawa observed that in perfused liver under hypoxia, decrease of oxidative phosphorylation activity is accompanied by decrease in the mitochondrial content of adenine nucleotides (8). Furthermore, the importance of intramitochondrial adenine nucleotides for oxidative phosphorylation activity has been pointed out by Asimakis and Aprille, who showed a reversible inactivation of oxidative phosphorylation by depletion of the nucleotides in isolated mitochondria (9).

In the present study, we found that adenine nucleotides were finally degraded to adenosine in mitochondria under anoxia, and that the nucleoside leaked out through the mitochondrial membranes. Thus, anoxia resulted in depletion of adenine nucleotides in mitochondria and concomitant decrease in oxidative phosphorylation capacity.

MATERIALS AND METHODS

Animals—Male Sprague-Dawley rats weighing about 200 g were used. They were starved overnight and then anesthetized by intraperitoneal injection of ketamine at 100 mg/kg body weight. Ischemic damage of mitochondria was produced by anoxic incubation of excised liver. To remove blood, livers were perfused with normal saline before excision. After the desired period of anoxic incubation at 37° C in normal saline, mitochondria were isolated and their respiratory control and adenine nucleotide contents were examined.

Preparation of Mitochondria—Mitochondria were isolated using 0.3 M mannitol containing 0.1 mM EDTA, pH 7.4, and 0.1% bovine serum albumin. Mitochondrial protein was determined by the method of Lowry *et al.* (10).

Measurement of Respiration and Adenine Nucleotide Contents—Mitochondria, 10–20 mg protein/ml, isolated from normal liver were incubated under various conditions in incubation medium consisting of 0.3 M mannitol, 10 mM Tris-HCl, pH 7.4, 5 mM potassium phosphate, 0.2 mM EDTA, 0.1% bovine serum albumin, and 1 mM MgCl₂. The rate of oxygen consumption was measured with a Clark-type electrode in incubation medium at 25°C with succinate as a respiratory substrate. Adenine nucleotides and purine metabolites were extracted from mitochondria with 0.5 N perchloric acid, and determined by high pressure liquid chromatography on an anion exchange column of IEX-540, and a cation exchange column of IEX-510, respectively, as described in the previous paper (7).

Separation of Mitochondria from External Medium—Mitochondria were separated from the incubation medium by the centrifuge column method (11). That is, the mitochondrial suspension was charged on a Sephadex G-50 column (in a disposable syringe, 1×5 cm, equilibrated with the incubation medium), the column was centrifuged at 2,000 rpm for 1 min, and the mitochondria separated from the medium were recovered in the effluent.

RESULTS

Mitochondrial Damage in Ischemic Liver-Previously we reported that the cellular level of ATP decreased sharply in ischemic rat liver and then the level of total adenine nucleotides decreased gradually (7). As shown in Fig. 1A, these levels in mitochondria were also found to decrease during ischemia, but their rates of decrease were a little different: in mitochondria, the content of total adenine nucleotides decreased faster than in whole cells and was almost zero after ischemia for 120 min, when the cellular level was still more than a quarter of the initial level (cf. Fig. 1 of Ref. 7). We also determined the levels of other mitochondrial components, including cytochromes, phospholipids, and matrix enzymes, such as aspartate aminotransferase and ornithine carbamoyltransferase. These other components did not change significantly in mitochondria during ischemia for up to 120 min (unpublished data). The loss of mitochondrial adenine nucleotides seems very serious for synthesis of ATP, because ADP is the sole phosphate acceptor in the oxidative phosphorylation system and intramitochondrial ATP is required for uptake of ADP by



Fig. 1. Decrease of adenine nucleotide contents in mitochondria isolated from ischemic liver. Mitochondria were isolated at 0°C from excised rat liver after anoxic incubation for 0, 15, 30, 60, and 120 min at 37°C. Mitochondria at 0 min were prepared immediately after excision of the liver from the experimental animal. Adenine nucleotide contents in mitochondria were measured without adding respiratory substrate (A) and after aeration with 5 mM succinate (B). \bullet , ATP+ ADP+AMP; \bigcirc , ATP; \blacksquare , ADP; \blacktriangle , AMP.

translocase, which is the only permeation system for adenine nucleotide in the mitochondrial inner membrane. Actually, when aerated in the presence of a respiratory substrate, such as succinate, all the ADP and AMP were converted to ATP in mitochondria prepared from excised liver incubated at 37°C under anoxic conditions for less than 60 min (Fig. 1B), whereas this conversion was not observed in mitochondria prepared from liver after ischemia for 120 min, which still contained small but significant amounts of all three adenine nucleotides. The activity of oxidative phosphorylation in mitochondria prepared from ischemic liver was also measured directly by adding ADP externally. As shown in Fig. 2, the respiratory control ratio decreased with decrease in the mitochondrial content of adenine nucleotide during ischemia and became unity after 120 min, indicating complete loss of phosphorylating capacity. This time was the same as that for loss of reversibility of the cellular ATP level in ischemic liver on recirculation (cf. Fig. 8 of Ref. 7).

Disappearance of Adenine Nucleotides in Isolated Mitochondria—A close relation between the capacity for oxidative phosphorylation and the mitochondrial contents of adenine nucleotides was also observed in mitochondria isolated from normal rat liver incubated *in vitro*. When the mitochondrial suspension was incubated at 25°C under anoxic conditions, loss of total contents of adenine



Fig. 2. Decrease of the respiratory control ratio (RCR) of mitochondria in ischemic liver. Data were obtained in the same experiments as for Fig. 1.



Fig. 3. Decrease of mitochondrial adenine nucleotide contents and accumulation of adenosine in the medium. Mitochondria, 14 mg protein/ml, isolated from normal liver were incubated at 25°C in the incubation medium. •, intramitochondrial ATP+ADP+AMP; \bigcirc , adenosine in the incubation medium; \blacktriangle , adenosine and its metabolites in the incubation medium; \blacksquare , respiratory control ratio.

nucleotides occurred in parallel to loss of phosphorylating capacity, expressed as the respiratory control ratio (Fig. 3). In the experiment demonstrating this, the rates of leakage of adenine nucleotides and matrix enzymes, such as aspartate aminotransferase and ornithine carbamoyltransferase, were also determined. None of these compounds was found in the external medium, but adenosine appeared in small amounts as its purine metabolites, such as inosine and hypoxanthine. The total amounts of the purine compounds found in the external medium fully accounted for the loss of nucleotides. Atractyloside or oligomycin, potent inhibitors of ATP/ADP translocase and F_0F_1 -ATPase, respectively, had no effect on the loss of nucleotides or the accumulation of purine compounds. These results indicate that the inner membrane barrier against diffusion of adenine nucleotides remained intact and that AMP accumulating inside the membranes was degraded to adenosine, which could permeate through the mitochondrial membranes. Neither ATP/ADP translocase nor F_0F_1 -ATPase is likely to be involed in this process. It seems likely that decrease in the level of total adenine nucleotides in mitochondria in ischemic liver proceeds in a similar manner to that observed in isolated mitochondria.

Protection of Oxidative Phosphorylation by Maintaining Adenine Nucleotide Levels-It is well known that the oxidative phosphorylation capacity is protected at 0°C, and also at higher temperatures, by the presence of either ATP or a respiratory substrate, such as 2-oxoglutarate or succinate, under aerobic conditions (12, 13). As shown in Fig. 4, the total adenine nucleotide content remained at a normal high level under conditions where the oxidative phosphorylation capacity could be maintained. On incubation of mitochondria in ice, the nucleotide level changed only slightly in 2 h (Fig. 4), more than 50% of the initial level remaining, and concomitantly the respiratory control ratio also remained almost unchanged for over 24 h (data not shown). When mitochondria were aerated at 25°C in the presence of succinate, there was no change in the nucleotide level, but a slight decrease in the respiratory control ratio in



Fig. 4. Maintenance of oxidative phosphorylation capacity and the level of total adenine nucleotides in mitochondria incubated under various conditions. Mitochondria, 14 mg protein/ml, isolated from normal liver were incubated in incubation medium: \blacksquare , at 0°C; \bullet , at 25°C; \blacktriangle , in the presence of 6 mM ATP at 25°C; \bigcirc , aerated by shaking with 6 mM succinate at 25°C. The level of total adenine nucleotides (A) and the respiratory control ratio (B) were measured.

2 h. This decrease was due to increase in the state 4 respiration rate, possibly caused by slight activation of ATPase. Added ATP was slowly hydrolyzed to ADP at 2.5 nmol/min/mg protein at 25°C. The decrease of respiratory control ratio when succinate or ATP was added was due to a slight increase in state 4 respiration, but does not imply a significant change of oxidative phosphorylation capacity. When a limited amount of ATP was added, its protective action decreased as hydrolysis proceeded, and ceased when it was exhausted (data not shown). ATP probably penetrated the mitochondria by antiport with ADP and was hydrolyzed by various ATP-consuming enzyme systems, because ADP was accumulated outside mitochondria and the protective action of ATP on maintenance of the total adenine nucleotide level was partially suppressed by atractyloside.

Absence of Protection by AMP-A high total adenine nucleotide level in mitochondria was shown to be very important for preservation of the oxidative phosphorylation capacity. Of the three species of adenine nucleotides, AMP had no effect without ATP or ADP, although it was readily converted to ADP in the presence of ATP. AMP accumulated in the mitochondria was hardly hydrolyzed to adenosine when Mg2+ and inorganic phosphate were omitted from the incubation medium, probably due to suppression of 5'-nucleotidase activity. Thus, when mitochondria were incubated under these conditions, both ATP and ADP were consumed, but the total adenine nucleotide level remained fairly high because of AMP accumulation. However, the oxidative phosphorylation capacity was not preserved at all under these conditions (Fig. 5). Since the enzyme inhibition was not complete, the adenine nucleotide level decreased a little, but the decrease was well matched by the amount of adenosine released outside. These results indicate that the protective action of adenine nucleotides on mitochondrial function is due to ATP and ADP, which are interconvertible by the action of adenylate kinase, and also by antiport with these compounds present outside the mitochondria.

Relation of the Level of ATP plus ADP with the Oxidative Phosphorylation Capacity of Mitochondria—The relation between the mitochondrial content of ATP plus ADP and the respiratory



Fig. 5. Decrease in the respiratory control ratio and accumulation of AMP inside mitochondria incubated in the absence of Mg^{2+} . Mitochondria, 9.9 mg protein/ml, isolated from normal liver were incubated at 25°C in incubation medium without Mg^{2+} and P_1 . \bullet , ATP+ADP+AMP; \bigcirc , ATP; \blacksquare , ADP; \blacktriangle , AMP; \square , respiratory control ratio.



Fig. 6. Correlation between the respiratory control ratio and the ATP+ADP content in various mito-[chondrial preparations. Mitochondrial preparations were obtained from normal and ischemic livers and from normal liver incubated under various conditions.

control ratio was examined by plotting the values for the two in various mitochondrial preparations isolated from normal and ischemic rat liver (Fig. 6). Respiratory control remained fairly constant at higher levels of ATP plus ADP than 5 nmol/ mg protein, but showed a very sharp drop at values of about 3 to 4 nmol. The ratio was always unity when the nucleotide level was lower than 2 nmol/mg protein. Since the minimal value for the nucleotide content can be accounted for by protein-bound nucleotides in mitochondria (11), it may be concluded that mitochondria lose phosphorylating capacity when free ATP and ADP in the matrix are exhausted. Rat liver mitochondria contain 15 to 20 nmol/mg protein of adenine nucleotide (14, 15). This level must be conserved not only in vivo but also in isolated mitochondria unless membranes of the organella are disrupted or the nucleotides are decomposed inside, because ATP/ADP translocase, which catalyzes equimolar exchange of these substrates, is the sole transport system for adenine nucleotides (16). Decrease in the mitochondrial nucleotide contents with concomitant loss of oxidative phosphorylation activity was first reported by Siekevitz and Potter, who also observed concomitant appearance in the external medium of materials with absorption at 260 nm (17). Later, Meisner and Klingenberg explained this as a result of leakage of adenine nucleotides from mitochondria (18). They observed leakage of radioactivity from mitochondria containing [14C]ATP, but did not identify the radioactive compound(s) in the medium. Nakazawa and Nunokawa reported that in perfused rat liver under hypoxic conditions mitochondria lose their oxidative phosphorylation function with concomitant decrease in their adenine nucleotide content (8). They also concluded that the decrease was due to leakage of the nucleotides caused by disruption of mitochondrial membranes under hypoxic conditions.

In contrast to these observations, it is demonstrated in this paper that the decrease in mitochondrial adenine nucleotide levels in vivo as well as *in vitro* is not attributable to their direct leakage, but to that of their degradation products, such as adenosine. The fact that adenosine was produced during anoxic incubation strongly suggests involvement of both adenylate kinase and 5'-nucleotidase in the degradation system in mitochondria, although the former enzyme has not been studied in mitochondrial matrix. Two isozyme species of adenylate kinase were reported to be localized in cytoplasm and intermembrane space of mitochondria (19). However, this did not necessarily imply its absence in the matrix, since residual activity (about 10%) remained in mitochondrial extracts after removal of the interspace isozyme by hypotonic treatment or immunoprecipitation (19). Participation of the enzyme activity in the matrix was also indicated by the observation in the

present study that AMP in mitochondria was readily converted to ATP by aerating mitochondria in the presence of succinate (Fig. 1). Occurrence and properties of mitochondrial 5'-nucleotidase has been described in rat liver by Greger and Fabianowska-Majewska (20). The mitochondrial enzyme is inhibited by ATP and has low affinity for AMP (20), although the cytoplasmic enzyme was reported to be activated by ATP (21). Thus, it is likely that adenosine cannot be produced in mitochondria until the blockage of oxidative phosphorylation is followed by consumption of ATP and subsequent accumulation of AMP. Adenosine produced inside leaks out, probably through a specific channel, and depletion of the mitochondrial level of ATP plus ADP can result in loss of oxidative phosphorylation activity because no ADP is available, even if the mitochondrial membrane system is unimpaired. Some energy-dependent process was assumed to be involved in the maintenance of the oxidative phosphorylation capacity (13). However, it seems likely that ATP inside mitochondria is involved in the ATP/ADP translocase system as an antiport substrate and need not be consumed for preservation of this capacity, since it can be well preserved even when intramitochondrial ATP and ADP are replaced by pyrophosphate (9).

The close correlation between the levels of intramitochondrial adenine nucleotides and oxidative phosphorylation capacity has been demonstrated in the elegant experiments of Asimakis and Aprille, in which the levels of ATP and ADP were reversibly changed by antiport with pyrophosphate, a substrate of ATP/ADP translocase (9). In the present investigation with the three species of adenine nucleotides, we also showed the importance of ATP plus ADP and the inefficacy of AMP for maintenance of oxidative phosphorylation capacity. At levels of more than 5 nmol/mg protein, the respiratory control ratio decreased slightly with decrease in the nucleotide levels, but the decrease became discontinuous below this level. The ratio was always unity at levels of less than 2 nmol/mg protein. Since a level of more than 1 is attributable to ATP and ADP tightly bound to F_0F_1 -ATPase (11), the value of 2 may correspond to protein-bound nucleotides. Thus, it may be concluded that the oxidative phosphorylation capacity of mitochondria is completely

lost when free ATP and ADP inside the mitochondria are exhausted.

Mitochondrial dysfunction has been explained by inhibition of ATP/ADP translocase as a result of acyl-CoA accumulation in ischemic tissue (3-5). In previous work, however, the translocase activity was determined by measuring the incorporation into mitochondria of ¹⁴C-labeled-ATP or -ADP added externally, not the intramitochondrial levels of nucleotides that exert decisive effects on both the quantity and rate of incorporation. Since the levels decrease sharply in ischemic tissues, such data do not seem reliable.

The leakage of adenosine from mitochondria seems to be facilitated by a specific channel, since the diffusion barrier of the inner membrane against various intramitochondrial substances was unimpaired even after loss of oxidative phosphorylation activity. Matrix enzymes, such as mitochondrial aspartate aminotransferase and ornithine carbamoyltransferase were observed not to be released for a while after loss of mitochondrial function either in ischemic liver or on incubation in vitro (Nishimura, T., unpublished data). Moreover, the intramitochondrial level of NAD plus NADH remained constant during anoxic incubation, insofar as these compounds were not decomposed to adenine compounds and nicotinamide (Watanabe, F., unpublished data).

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