CRYOBIOLOGY 22, 111–118 (1985)

Functional Changes in Mitochondrial Properties as a Result of Their Membrane Cryodestruction

II. Influence of Freezing and Thawing on ATP Complex Activity of Intact Liver Mitochondria

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The influence of the freeze-thawing rates on ATP synthetase (ATPase) complex of intact liver mitochondria was investigated. It was shown that the increase in latent ATPase activity and decrease in ATP synthetase activity resulted from an influence on the inner mitochondrial membrane. An increase in freeze–thawing rates led to the preservation of ATP synthetase activity and ATP hydrolysis reduction. Kinetic parameter changes of the ATP synthetase reaction resulted from an insignificant nonspecific increase in the inner mitochondrial membrane permeability and changes in its electrochemical potential level.

The transmembrane electrochemical potential of hydrogen ions generated from a respiratory chain is a driving force for ATP synthesis from ADP and nonorganic phosphate in the mitochondria (15, 16). This is accomplished by the ATP synthetase complex of mitochondria. The activity of this complex is directly under the control of the mitochondrial membrane. This affects the reaction of ATP synthesis in perfectly isolated mitochondria. In cases leading to uncoupling of oxidation from phosphorylation, however, this complex accomplishes the reverse reaction—ATP hydrolysis. A typical case in point is an increase in ATPase activity as a result of uncoupling at membrane destruction (10, 11, 17). Therefore, the examination of the activity of this complex is an appropriate method to evaluate the changes in membrane properties after cryotreatment and cryoinjury. The basic aim of this study was to examine, simultaneously, the ATP synthetase and ATPase reactions of intact liver mitochondria after freeze–thawing at different rates.

Received March 9, 1984; accepted August 14, 1984

MATERIALS AND METHODS

Intact liver mitochondria were isolated according to the method of Johnson and Lardy (12). ATPase activity was defined as a change in the hydrogen ion concentration during the reaction (4) in a medium of 0.15 *M* sucrose, 75 m*M* KCl, 0.1 m*M* EDTA, 5 μ*M* rotenone, 1 m*M* ATP, and 5 m*M* KH_2PO_4 (pH 7.5).

ATP synthetase activity was also monitored by the change in concentration of H^+ ions in a reaction medium of 0.15 *M* sucrose, 75 m*M* KCl, 0.1 m*M* EDTA, 5 μ*M* rotenon, 0.2 m*M* ADP, and 5 m*M* KH_2PO_4 (pH 7.5).

Freezing and thawing of samples. Isolated mitochondria were suspended in 0.25 *M* sucrose (0.2 ml suspension), frozen in liquid nitrogen, and thawed at various rates (21). The oxygen consumption velocity was polarographically measured with a Clark-type electrode (5, 6) in a reaction medium of 0.15 *M* sucrose, 75 m*M* KCl, 0.1 m*M* EDTA, 5 μ*M* rotenone, and 5 m*M* KH_2PO_4 (pH 7.5). Protein concentration was determined by the Bio-Rad method according to Bradford (2, 3).

0011-2240/85 $3.00

The kinetic parameters, i.e., Michaelis constant (K_m) and maximum velocity (V_{max}) of the ATP synthetase and ATPase reactions were obtained using regression analysis. Experimental data were processed and compared by analysis of variance. All calculations and drawings were carried out by means of a computer (HP 9825A), plotter (HP 9872C), statistics software (HP 9825: vol. 1, No.09825-15001; vol.2, part No.09825-15011), and with the aid of local programs.

RESULTS AND DISCUSSION

It was shown in earlier studies that the property changes of the inner mitochondrial membrane are bound up with freezing and thawing rates (21). The changes at rapid freeze–thaw rates were much more insignificant than the changes at slow freeze–thaw rates. Similar results are also presented in other papers related to the influence of freezing rates on oxidative phosphorylation (1, 7, 19).

Typical curves showing the oxygen consumption of liver mitochondria before and after freezing (750°C/min) and subsequent thawing (510°C/min) are illustrated in Fig. 1. In mitochondria treated at low temperature, a particular preservation of the respiratory control and a particular transition to the forth state was observed (Fig. 1, curve 2).

The data in Table 1 show the velocity changes of the oxygen consumption in different states. At optimal low-temperature treatments the oxygen consumption increased three times in second state, insignificant changes in third state (after ADP addition), and increased 3.5 times in fourth state. The respiratory control index, determined accordingly to Lardy (RCI = 1.37),

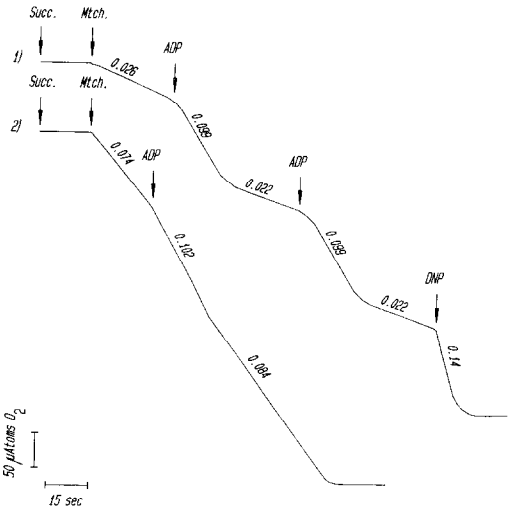

FIG. 1. Influence of rapid freeze–thawing on the oxidative phosphorylation of intact liver mitochondria. Curve 1, control sample; curve 2, rapid freeze–thawed mitochondria (750–510°C/min). The arrows point to the additions: Mtch. mitochondria (4.4 mg protein); Succ., 5 m*M* succinate; ADP, 0.2 m*M*; DNP, 0.08 m*M* 2,4-dinitrophenol. On the slopes are given the rates of oxigen consumption in different states (μAtoms O_2 min^{-1} mg $protein^{-1}$). For reference on the reaction medium see Materials and Methods.

TABLE 1
Influence of Rapid Freeze–Thawing (750–51°C/min) on Oxygen Consumption

Treatment	V_{St2}	V_{St3}	V_{St4}	RCI	RCR	ADP:O
Control sample	0.026 ± 0.008*	0.099 ± 0.03	0.0217 ± 0.009	4.51 ± 0.3	4.7 ± 0.4	1.7 ± 0.14
Freeze–thawing (750–510°C/min)	0.074 ± 0.02*	0.102 ± 0.02	0.085 ± 0.02	1.37 ± 0.19	1.2 ± 0.09	1.3 ± 0.05

Note. Oxygen consumption velocity in different states, μAtoms O_2 min^{-1} mg $protein^{-1}$; RCI, respiratory control index—Lardy; RCR, respiratory control ratio—Chance; and ADP:O of intact liver mitochondria. For reference on the reaction medium see Materials and Methods.

* Standard deviation. $P \geq 0.001$, $n = 15$.

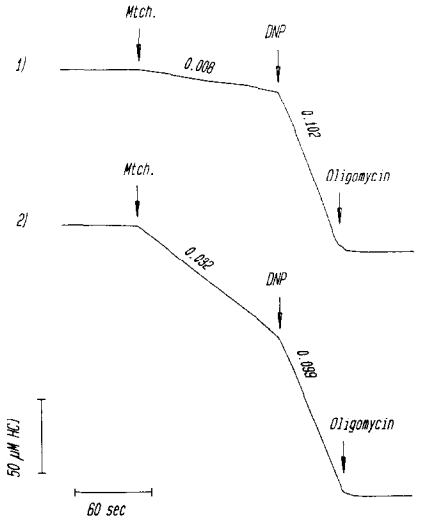

FIG. 2. Influence of rapid freeze–thawing on the ATPase activity of intact liver mitochondria. Curve 1, control sample; curve 2, rapid freeze–thawed mitochondria (750–510°C/min). The arrows point to the additions: Mtch., mitochondria (4.9 mg protein); DNP, 0.08 m*M* 2,4-dinitrophenol; Oligomycin (1 μg/mg protein). On the slopes are given the rates of the enzyme reaction (μg ion H^+ min^{-1} mg $protein^{-1}$). For reference on the reaction medium see Materials and Methods.

was higher than that determined accordingly to Chance (RCR = 1.19), which was probably connected with activation of ATPase.

It is obvious from Fig. 2 and data shown in Table 2 that, after rapid freeze–thawing, there was a partial stimulation of the latent ATPase activity, which was completely released after addition of 2,4-dinitrophenol (DNP), an uncoupler.

After a similar low-temperature treatment, the mitochondrial preparations were able to realize ATP synthesis (Fig. 3). Exogenously added ADP (0.2 m*M*) was completely phosphorylated without carrying into effect the reverse reaction—ATP hydrolysis (Fig. 3, curve 1). As could be seen (Fig. 3, curve 4), these mitochondria hydrolyzed exogenously added ATP at a slow rate. Therefore, presumably the mitochondria use exogenously added ATP (Fig. 3, curve 4) and *de novo*-synthesized ATP (Fig. 3, curve 1) in different ways. A likely reason for the absence of hydrolysis of *de novo*-synthesized ATP could be the presence of succinate in the reaction medium (Fig. 3, curve 1). Under these conditions the respiratory chain is in continuous action and probably compensates for the proton leakage through the membrane. If that statement is true, it is most likely that the suppressing of the respiratory chain activity would lead to hydrolytic stimulation of the newly synthesized ATP. Indeed, the inhibition of the respiratory chain with antimycin A after the complete ADP phosphorylation led to a partial stimulation of the ATPase activity, i.e., the ATPase was striving for compensation of the membrane hydrogen potential disturbance (Fig. 3, curve 2). On the other hand, the succinate addition into the reaction medium completely inhibited the ATPase activity of the

TABLE 2
Influence of Rapid Freeze–Thawing (750–510°C/min) on the ATPase Activity of Intact Liver Mitochondria

Treatment	ATPase activity (μg ion H^+ min^{-1} mg $protein^{-1}$)		Stimulation
	−DNP	+DNP	
Control sample	0.008 ± 0.001*	0.102 ± 0.03	—
Freeze–Thawing (750–510°C/min)	0.032 ± 0.012*	0.099 ± 0.02	3.09

Note. For reference on the reaction medium see Materials and Methods.
* Standard deviation. $P \geq 0.001$; $n = 20$.

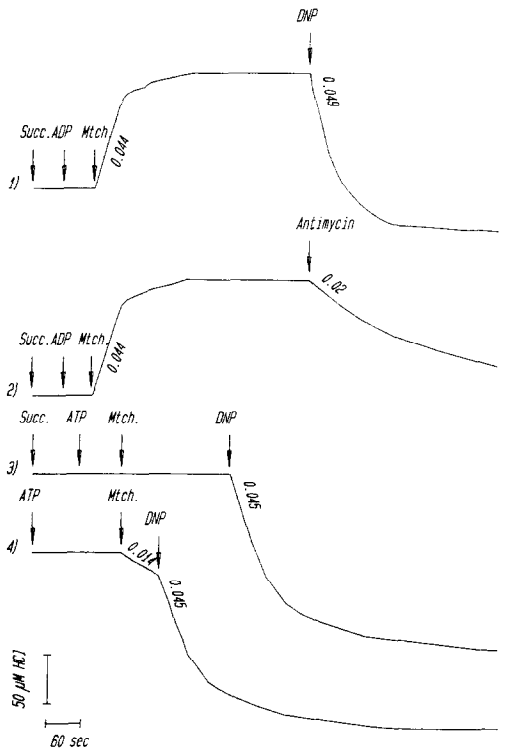

FIG. 3. Influence of rapid freeze–thawing on the ATP synthetase and ATPase activities. Curve 1, ATP synthesis in freeze–thawed (750–510°C/min) mitochondria; curve 2, hydrolysis stimulation of the synthesized ATP in low temperature treated mitochondria after respiratory chain inhibition; curve 3, ATPase activity inhibition from succinate rapid freeze-thawed mitochondria; curve 4, ATPase activity in freeze-thawed (750–510°C/min) mitochndria. The arrows point to the additions: ADP or ATP, 0.2 m*M;* Succ. 5 m*M* succinate; Mtch., mitochondria (5.4 mg protein); DNP, 0.08 m*M* 2,4-dinitrophenol; Antimycin (2 μg/mg protein). On the slopes are given the rates of the enzyme reactions (μg ion H^+ min^{-1} mg $protein^{-1}$). The oxidative phosphorylation (curves 1 and 2) was monitored in a reaction medium of 0.15 *M* sucrose, 75 m*M* KCl, 0.1 m*M* EDTA, 5 μ*M* rotenone, 5 m*M* KH_2PO_4 (pH 7.5). The ATP activity was monitored in the same reaction medium.

mitochondria treated at low temperature, monitored in the same medium as for the ATP synthesis with 0.2 m*M* exogenously added ATP (Fig. 3, curve 3).

The inhibition of the ATPase activity after succinate addition (Fig. 3, curve 3) and stimulation of the hydrolysis of the *de novo*-synthesized ATP after inhibition of the electron flow (Fig. 3, curve 2) allowed the conclusion that uncoupling of the phosphorylation from oxidation after freeze–thawing at high rates results from the insignificant destruction of the inner mitochondrial membrane and increase in the nonspecific permeability for hydrogen ions.

In Fig. 4 the relationships of released ATPase and saved ATP synthetase activities dependence on freezing rate at a constant thawing rate are shown. The increase in freezing rate results in a higher phosphorylation rate and a lower ATPase activity before DNP addition. Hence, the high freezing rates partially save the native mitochondrial structure, which determines ATP synthetase activity.

Saving of the structural and functional integrity of the membrane is dependent upon the thawing velocity as well (Fig. 5). Preservation of the membrane properties which determine ATP synthetase activity (the phosphorylation rate is saved by more than 80%, curve 2, and the latent ATPase activity is released only by 25%, curve 1) is higher at high thawing rates than at low thawing rates (ATPase activity is released by 100%).

The equal influence of freezing and thawing rates shows that the cryoinjury results from a certain membrane state (perhaps phase transition of lipids) the preparation is running through at freezing as well as at thawing. The duration of a stay in that state is determined by freeze–thawing rates. The prolonged sojourn in that unusual state (slow freeze–thawing rates) causes a complete uncoupling and, vice versa, high freeze–thawing rates lead to a partial uncoupling. Therefore, the cryodamage is probably due to relatively slow processes, compared with the freeze–thawing process, which are not completely developed at high freeze–thawing rates.

The results presented above indicate that the variations in ATPase activity are connected with changes in the structure of the

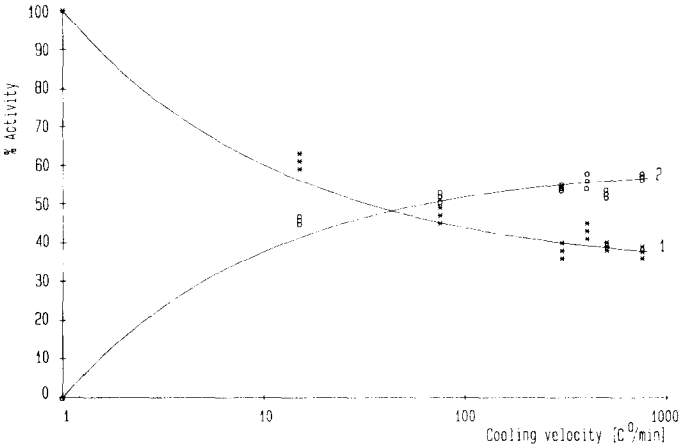

FIG. 4. Influence of different freezing rates at constant thawing rates (510°C/min) on the Curve 1, released ATPase activity, and curve 2, preserved ATP synthetase activity. ATPase activity (100%) is the activity of uncoupled intact mitochondria with 0.08 m*M* DNP, 0.106 μg ion H^+ min^{-1} mg $protein^{-1}$. The reaction mediun contained 1 m*M* ATP. ATP synthetase activity (100%) is the activity of intact mitochondria, 0.108 μg ion H^+ min^{-1} mg $protein^{-1}$. The reaction medium contained 0.2 m*M* ADP. The samples contained 5.43 mg protein. For reference on the reaction medium see Materials and Methods.

inner mitochondial membrane due to the nonspecific destruction and increase in the permeability for hydrogen ions without any other substantial changes. Therefore, presumably after cryoinjury, the maximum velocity of the ATPase reaction would be increased without any changes in the apparent Michaelis constant, K_m (lowering of the membrane barrier would lead to an increase in maximum velocity without any in-

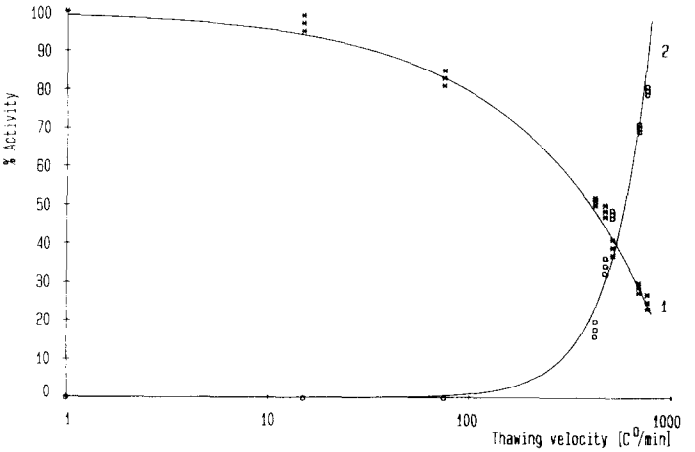

FIG. 5. Influence of different thawing rates at constant freezing rates (750°C/min) on the curve 1, released ATPase activity, and curve 2, preserved ATP synthetase activity. ATPase activity (100%) is the activity of uncoupled intact mitochondria with 0.08 m*M* DNP, 0.106 μg ion H^+ min^{-1} mg $protein^{-1}$. The reaction mediun contained 1 m*M* ATP. ATP synthetase activity (100%) is the activity of intact mitochondria, 0.108 μg ion H^+ min^{-1} mg $protein^{-1}$. The reaction medium contained 0.2 m*M* ADP. The samples contained 5.43 mg protein. For reference on the reaction medium see Materials and Methods.

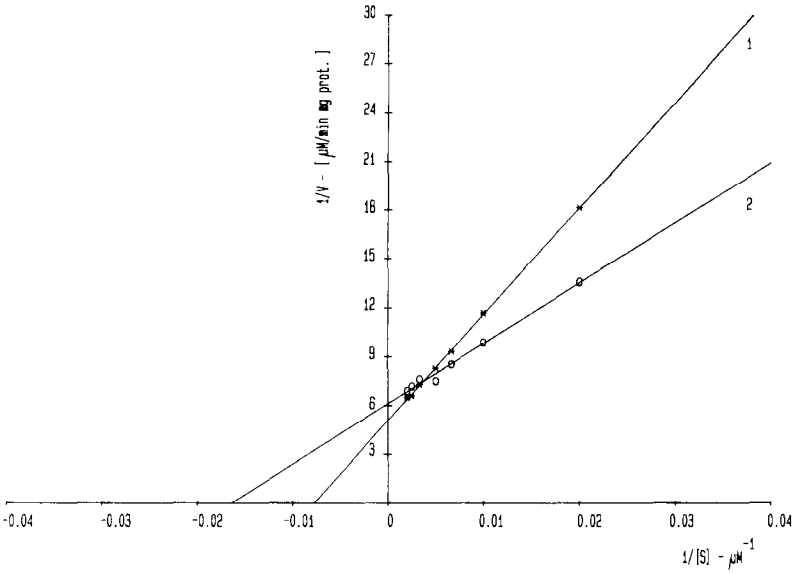

FIG. 6. Influence of freeze–thawing of intact liver mitochondria on the ATPase reaction velocity (Lineweaver–Burk plot). Curve 1, control sample; curve 2, freeze–thawed 750–510°C/min) mitochondria. The ATP activity of the control and freeze–thawed mitochondria was monitored in a reaction medium of 0.15 *M* sucrose, 75 m*M* KCl, 0.1 m*M* EDTA, 5 μ*M* rotenone, 5 m*M* KH_2PO_4, and increasing concentrations of ATP (0.05, 0.1, 0.15, 0.2, 0.3, 0.4, and 0.5 m*M*) (pH 7.5), and addition of 0.08 m*M* 2,4-dinitrophenol. Protein in the sample was 4.3 mg.

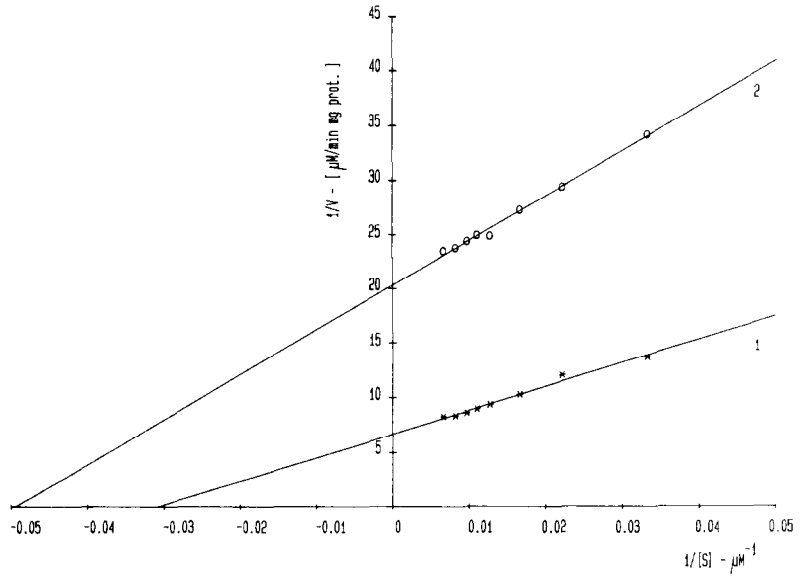

FIG. 7. Influence of freeze–thawing of intact liver mitochondria on the ATP synthetase reaction velocity (Lineweaver–Burk plot). Curve 1, control sample; curve 2, freeze–thawed (750–510°C/min) mitochondria. The ATP synthetase activity of the control and freeze–thawed mitochondria was monitored in a reaction medium of 0.15 *M* sucrose, 75 m*M* KCl, 0.1 m*M* EDTA, 5 μ*M* rotenone, 5 m*M* succinate, 5 m*M* KH_2PO_4 and increasing concentrations of ADP (0.03, 0.045, 0.06, 0.08, 0.09, 0.1, 0.12, and 0.15 m*M*) (pH 7.5). Protein in the sample was 4.3 mg.

fluence on the enzymes carrying out the hydrolysis).

Investigation of the ATPase kinetic parameters showed that, after freeze-thawing, the maximum velocity of ATPase reaction changed insignificantly, but there was significant change in K_m, which was lowered two times in comparison with the kinetic parameters of the control mitochondria (Fig. 6). Hence, the changes in ATPase activity are not only connected with changes in hydrogen ion permeability of the membrane.

Regardless of the wide differenses in K_m values for ATPase reaction catalyzed from various preparations (intact mitochondria, submitochondrial particles, isolated ATPase), it may be concluded, based upon other investigations, that the structural destruction of the inner mitochondrial membrane does not lead to rapid changes in the K_m values. The average value of K_m is in the range 0.1–0.3 m*M* (9, 14, 18, 20, 22), which shows that the membrane destruction does not exert influence on factor F_1, which directly caused ATP hydrolysis.

Simultaneous with the changes in kinetic parameters of the ATPase activity, changes were observed in kinetic parameters of the phosphorylation reaction (decrease in V_{max} and K_m two times for ADP, Fig. 7).

As it was shown, the membrane destruction after high freeze–thawing rates was insignificant (Fig. 3, curve 1, and Fig. 5, curve 2) and, therefore, the membrane maintains the necessary potential for carrying out ATP synthesis following exogenous addition of ADP. Presumably, the observed changes in ATP synthetase kinetic parameters are connected with certain changes in the electrochemical potential, which is sufficient for ATP synthesis but not the same value corresponding to that of intact, nontreated mitochondria. This assumption could be interpreted with results shown earlier in the literature (13) and considerations (8) of the relation between the membrane potential value and the kinetic constants of the phosphorylation reaction.

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