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Activity of ATP Synthetase Complex after Low Temperature Treatment or Freeze-Drying of Mitochondria Isolated from Skeletal Muscles

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The influence of freezing, thawing, or freeze-drying on ATP synthetase complex of isolated skeletal muscle mitochondria was studied. Cooling to −60 or to −196°C and rapid thawing did not change activity significantly. Slow warming stimulated the release of latent ATP-ase activity and decreased ATP synthesis. These changes were more pronounced after freeze-drying.

Mitchell's chemiosmotic hypothesis of the mechanism of oxydative phosphorylation is based upon the presence of an ATP synthetase complex in the inner mitochondrial membrane (7, 8). The activity of this complex depends on the integrity of the membrane. Injuries affecting the membrane as a result of various treatments, including cryoprocessing or freeze-drying, could be estimated by the change of ATP synthesis or hydrolysis rate, since these reactions are catalyzed by the complex.

Experiments with isolated rat liver mitochondria show that slow freezing and thawing lead to an increase of latent ATP-ase activity and to a decrease of ADP phosphorylation rate (12). The same effects are observed after slow freezing rapid thawing (10). Freeze-drying of rat liver mitochondria in the presence of trehalose leads to partial preservation of inner mitochondrial membrane integrity (13). Trehalose proves to be quite efficient for preservation of sarcoplasmic reticulum after freeze-drying (3, 4).

In this study the changes of the activity of ATP synthetase complex after freezing or freeze-drying of skeletal muscle mitochondria are investigated.

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MATERIALS AND METHODS

Intact skeletal muscle mitochondria are isolated from pig musculus soleus by the method of differential centrifugation in Chappell–Perry's medium (2). After isolation mitochondria are resuspended in 0.25 *M* sucrose solution containing 0.1% beef serum albumin. Polysep's effect (Polysep, Pharmachim, Bulgaria) on mitochondrial recovery after freezing or freeze-drying is estimated in 5, 10, and 15% (v/v) final concentration.

Samples of 0.25 ml in plastic vials are frozen to −60°C (refrigerator) or to −196°C (liquid nitrogen).

Fast thawing is performed in a water bath at 37°C and slow warming in air at ambient (22°C) temperature.

Freeze-drying is performed in a GT-1 "Leybold heraeus" laboratory apparatus. The samples are rehydrated with deionized water.

ATP-ase activity is registered following the pH changes of the reaction medium (15 m*M* sucrose, 75 m*M* KCl, 0.1 m*M* EDTA, 5 m*M* KH_2PO_4, and 1 m*M* ATP, pH 7.5). When ATP synthesis is measured ADP (200 μ*M*) is added in the reaction medium instead of ATP (1).

The kinetic parameters of ATP synthetase reaction are determined by regression analysis, and the protein content of the

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samples is determined by the method of Lowry (6).

RESULTS AND DISCUSSION

The influence of freezing and thawing on ATP-ase activity is represented in Figs. 1 and 2. The isolation of mitochondria leads to release of very weak ATP-ase activity (control value). The reaction is stimulated after the addition of DNP. When oligomycin is added to the medium the ATP-ase is completely suppressed.

After freezing to −60 or to −196°C and fast thawing a partial stimulation of the latent ATP-ase activity is observed: 2.5 times for the first freezing rate and 3.5 times for the second one (curve 2, Figs. 1 and 2), and a maximum value is reached after the addition of DNP to the medium.

Slow warming rates lead to a considerable release of latent ATP-ase activity (curve 3, Figs. 1 and 2). Slow warming is more injurious when mitochondria are frozen to −196°C. Accordingly, addition of DNP leads to a lower stimulation of hydrolysis reaction.

Warming rates affect mitochondrial

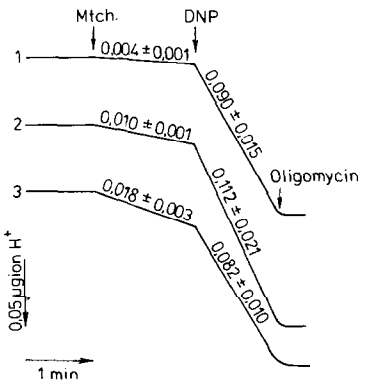

FIG. 1. ATP-ase activity of −60°C frozen skeletal muscle mitochondria, thawed after 24 hr. 1, Control mitochondria; 2, fast thawing; 3, slow thawing. The composition of the medium is described under Materials and Methods. Arrows indicate the addition of mitochondria (Mtch.) 1.37 mg protein; dinitrophenol (DNP) 0.08 m*M*; oligomycin, 1 μg/mg protein. Numbers denote enzyme reaction rates, μg ion H^+/min/mg protein.

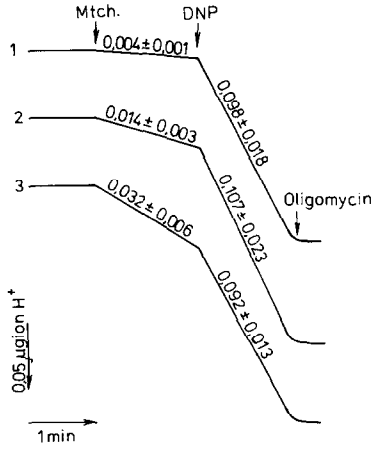

FIG. 2. Influence of freezing in liquid nitrogen on the ATP-ase activity of skeletal muscle mitochondria. The nomenclature and additives are as in Fig. 1.

ability for ATP synthesis in an analogous manner, fast thawing rate being more favorable (curves 2a, 3a, Fig. 3). After the exhaustion of ADP no hydrolysis of *de novo* synthesized ATP takes place. Slow warming is more injurious to mitochondria frozen to −196°C.

These data indicate that slow thawing results in a considerable injury of the inner mitochondrial membrane. Probably, as we can judge from previous studies in our laboratory of rat liver mitochondria and from results reported by other authors, the changes of the activity of the ATP synthetase complex are due to an increase in the proton permeability of the membrane and a decrease of the membrane electrochemical potential (11–13).

It is well known that an increased proton translocation through the membrane always leads to an uncoupling of oxidation from phosphorylation (9), which results in lowered rate of ATP synthesis, increased ATP-ase activity in the absence of the uncoupling agent, and reduction in its stimulating action on ATP-ase reaction. A possible result of the cooling–slow warming process could be the changes in the ATP-synthetase complex itself, namely, in its

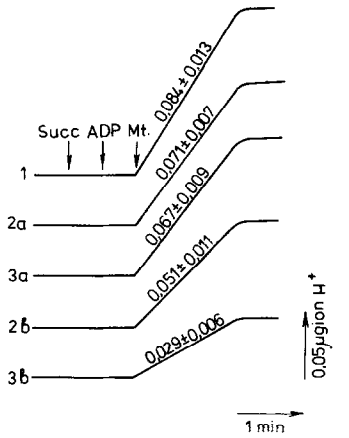

FIG. 3. Influence of the freezing–thawing regime on the ATP synthetase activity of skeletal muscle mitochondria. 1, Control mitochondria; 2, −60°C frozen mitochondria; 3, −196°C frozen mitochondria; a, fast thawing; b, slow thawing. The composition of the reaction medium is given under Materials and Methods. Arrows indicate the additives: succinate, 5 m*M*; ADP, 200 μ*M*; mitochondria, 1.37 mg protein. Numbers denote enzyme reaction rates, μg ion H^+/min/mg protein.

structure and location in the membrane, which could be the cause of its activity changes (3). Slow thawing is less injuring following freezing down to −60°C.

We studied the kinetic parameters of ADP phosphorylation after freezing and thawing. The kinetic parameters change significantly. When frozen to −196°C mitochondria are thawed slowly, V_{max} decreases about three times (0.090 μg ion H^+/min/mg protein for control mitochondria and 0.030 μg ion H^+/min/mg protein for cryotreated mitochondria; Fig. 4). Changes are observed also in K_m: 12.4 μ*M* for controls and 22.5 μ*M* for cryoprocessed mitochondria. Kayalar observed the same effect on V_{max} and K_m for ADP, resulting from treatment with the uncoupler DNP (5). The latter produces a reduction in membrane electrochemical potential. Freezing and thawing have the same result

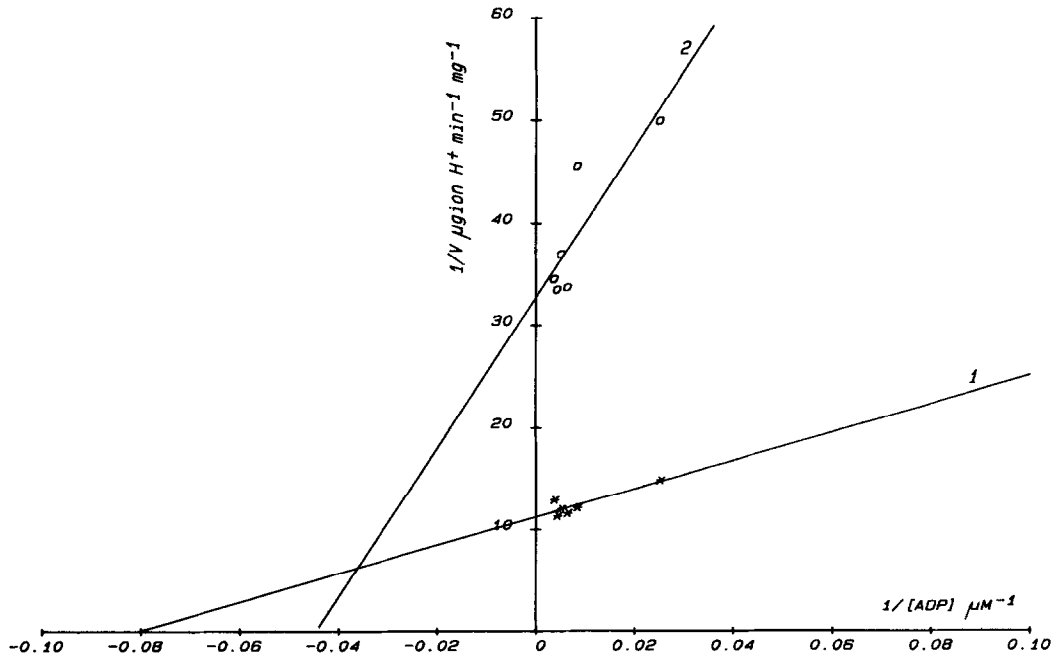

FIG. 4. Influence of freeze–thawing of intact skeletal muscle mitochondria on the ATP synthetase reaction rate (Lineweaver–Burk plot). 1, Control mitochondria; 2, −196°C frozen mitochondria, thawed slowly. The ATP synthetase activity was monitored in a reaction medium of 15 m*M* sucrose, 75 m*M* KCl, 0.1 m*M* EDTA, 5 m*M* succinate, 5 m*M* KH_2PO_4, and increasing concentrations of ADP (0.04, 0.12, 0.16, 0.20, 0.24, 0.28 m*M*) (pH 7.5). Protein in the sample was 1.37 mg.

TABLE 1
ATP-ase Activity of Skeletal Muscle Mitochondria – 196°C Frozen and Freeze-Dried in the Presence of Different Concentrations of Polysep

Mitochondria processing	Composition of suspending medium	ATP-ase activity μg ion H^+/min/mg protein		
		−DNP	+DNP	Stimulation
Control mitochondria	0.25 *M* sucrose	0.005 ± 0.002	0.108 ± 0.029	24.13 ± 4.32
Fast-frozen-thawed mitochondria	0.25 *M* sucrose	0.013 ± 0.006	0.111 ± 0.043	8.71 ± 2.07
	0.25 *M* + 5% Pol.	0.013 ± 0.005	0.123 ± 0.021	9.62 ± 1.63
	0.25 *M* + 10% Pol.	0.012 ± 0.005	0.118 ± 0.057	9.82 ± 2.75
	0.25 *M* + 15% Pol.	0.013 ± 0.004	0.099 ± 0.015	9.24 ± 1.73
Freeze-dried-rehydrated mitochondria	0.25 *M* sucrose	0.023 ± 0.011	0.037 ± 0.012	1.64 ± 0.26
	0.25 *M* + 5% Pol.	0.019 ± 0.006	0.042 ± 0.016	2.22 ± 0.55
	0.25 *M* + 10% Pol.	0.018 ± 0.007	0.038 ± 0.011	2.09 ± 0.57
	0.25 *M* + 15% Pol.	0.017 ± 0.007	0.033 ± 0.008	2.01 ± 0.51

due to altered membrane permeability. Probably there is a relationship between registered changes in ATP synthetase kinetic parameters and reduced membrane electrochemical potential after cryoprocessing. This could be better explained following the considerations of Kayalar (5).

Freezing is the first step in freeze-drying. Greater changes in ATP synthesis and hydrolysis rates are observed after lyophilization and rehydration (Table 1). The level of released latent ATP-ase activity reaches 4–5 times the control value, while the rate of ATP hydrolysis reaction registered after the addition of DNP to the medium decreases about three times compared to the control value. The fact that some, although very low, DNP stimulated ATP-ase activity in freeze-dried mitochondria is observed shows that the inner membrane is not totally destroyed. Polysep has some protective effect on the inner mitochondrial membrane during freeze-drying.

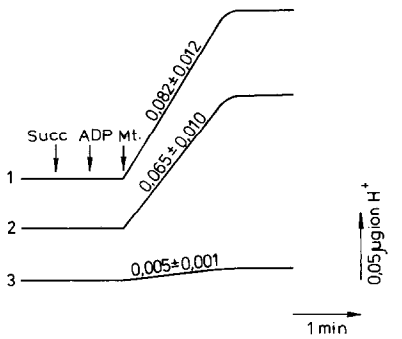

FIG. 5. Influence of lyophilization–rehydration on the ATP synthetase activity of skeletal muscle mitochondria. 1, Control mitochondria; 2, – 196°C frozen mitochondria, thawed quickly; 3, 0.25 *M* sucrose lyophilized mitochondria. The composition of the reaction medium is described under Materials and Methods. Arrows indicate the additives: Succ., 5 m*M* succinate; ADP, 0.2 m*M*; Mt., mitochondria, 1.69 mg protein. Numbers denote enzyme reaction rates, μg ion H^+/min/mg protein.

ATP synthesis is severely suppressed after freeze-drying. The phosphorylation rate is about 6% of the control value (curve 3, Fig. 5). This indicates that the inner mitochondrial membrane is severely damaged during freeze-drying. After 1 week and 1 month storage of the lyophilized product, the established changes of the synthesis and hydrolysis reaction rates of the ATP molecule remain unaltered.

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