REVERSAL OF THE EFFECTS OF FREEZING ON OXIDATIVE PHOSPHORYLATION IN THE MYCOBACTERIUM PHLEI SYSTEM

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Received March 17, 1971

SUMMARY: It was previously shown that exposure of electron transport particles from Mycobacterium phlei to heat (50°) for 10 min increased the level of phosphorylation and eliminated the requirement for soluble coupling factors. In contrast, particles subjected to freezing followed by slow thawing exhibited decreased P:O ratios. This loss in activity was restored following heat-treatment of the frozen particles. Mixture of ATP and Mg^{++}, soluble coupling factors and cryoprotective agents like glycerol and dimethyl sulfoxide protected the ETP from the effects of freezing.

It was shown earlier that heat-treatment of the electron transport particles (ETP) from Mycobacterium phlei resulted in increase in the level of phosphorylation coupled to oxidation of substrates (1). In addition, heat-treatment eliminated the requirement of soluble coupling factors (2). However, the heat-treated ETP required "particle-bound" coupling factor(s) for restoration of phosphorylation. These results could be explained by assuming that heat-treatment (a) removed a heat-labile natural inhibitor(s), or (b) brought about structural alteration of the membrane (1, 3). The presence of an inhibitor(s) of oxidative phosphorylation is in line with the demonstration that an ATPase inhibitor is present in mammalian mitochondria (4, 5, 6) and chloroplasts (7). These inhibitors were destroyed by heat-treatment (6, 7). However, attempts to isolate such an inhibitor from M. phlei ETP have not been successful. In the present studies attempts have been made to alter the membrane structure and to determine the effects of such alteration on oxidative phosphorylation.

MATERIALS AND METHODS: The growth conditions and harvesting of M. phlei ATCC 354 and the preparation of ETP have been described earlier (2, 8). The ETP were suspended in 0.15 M KCl and subjected to heat-treatment at 50° for 10 min (1). The ETP were frozen rapidly by freezing (a) in dry ice-acetone slurry (-75°) for 10 min, or (b) in liquid nitrogen (-190°) for 2 min. The frozen ETP were thawed slowly at room temperature.

Oxygen uptake was measured with the Gilson differential respirometer at 30°. Inorganic orthophosphate was measured using the method of Fiske and SubbaRow (9). Protein was determined either by the Biuret method (10) or, by the method of Stadtman et al (11).

RESULTS AND DISCUSSION: The mechanism by which heat-treatment brings about increased phosphorylation in M. phlei ETP has not been understood. The possibility of an alteration in the membrane structure during heat-treatment was studied. It is well known that freezing and thawing results in structural alteration of the membranes leading to changes in the activity of membrane bound enzymes (12, 13). In the mammalian mitochondrial and M. phlei extracts, oxidative phosphorylation was shown to be lost on freezing and thawing due to damage to the structural organization of membranes (14, 15, 16, 17). Thus, the effect of freezing and thawing on oxidative phosphorylation in ETP from M. phlei was studied. Freezing of the ETP at -75°C was found to decrease phosphorylation by about 50% without significant effect on oxidation (Figure 1). However, freezing in liquid nitrogen resulted in a significant decrease in both oxidation and phosphorylation.

It is interesting to note that the decreased phosphorylation brought about by freezing (-75°) was partially restored by heating the ETP (Table 1). Since in some experiments the damage caused by liquid nitrogen, was irreversible, ETP were only used following exposure to -75°. Prior heating also afforded a certain degree of protection to phosphorylation. This effect was further enhanced when succinate was used as substrate. There was a decrease in phosphorylation (38%) when the heat-treated ETP were frozen; nevertheless the

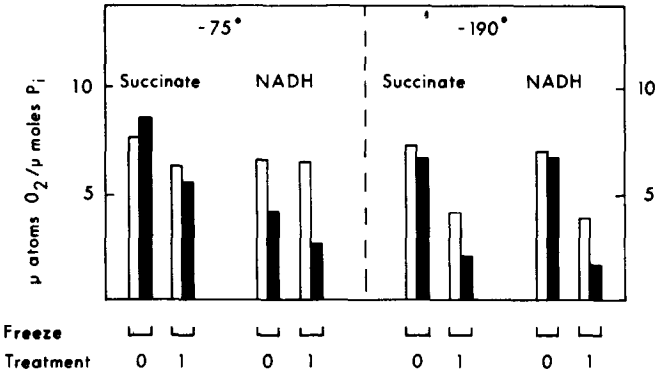

Figure 1. Effect of freezing the ETP on oxidative phosphorylation. The reaction mixture consisted of ETP (2.0 mg), 100 μmoles HEPES-KOH buffer pH 7.4, 50 μmoles glucose, 15 μmoles orthophosphate, 30 μmoles $MgCl_2$, 3.0 mg hexokinase, 2.5 μmoles AMP. The substrates used were succinate 100 μmoles or NADH generated by 100 μmoles of ethanol in presence of 0.5 mg alcohol dehydrogenase and 1.0 μmole NAD^+ and 25 μmoles hydrazine (to trap the aldehyde formed). The final volume was made up to 2.0 ml. The reaction was run at 30°C for 15 min when generated NADH was used as oxidant and 25 min when succinate was used as substrate, and was stopped by the addition of 1.0 ml 10% TCA. The samples were centrifuged and aliquotes of the supernatant were used for phosphate determination. ETP were frozen either at -75° (dry ice in acetone) for 10 min or -190° (liquid nitrogen) for 2 min. The closed bars represent the phosphorylation, while the open bars represent oxidation.

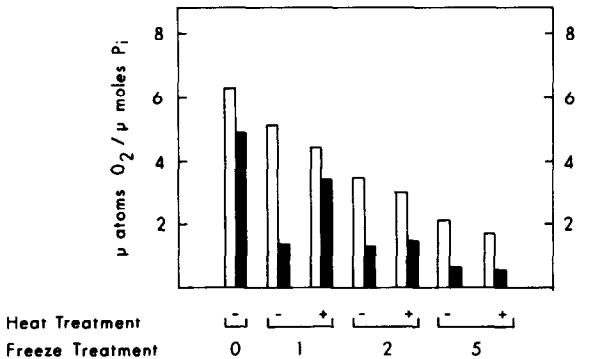

Figure 2. Effect of repeated freezing and thawing on the ETP. Conditions similar to those described under Figure 1. Generated NADH was used as substrate.

TABLE 1

Effect of Freezing and Subsequent Heating the ETP on Oxidative Phosphorylation

Substrate	Treatment	Oxygen uptake μatoms	Pi esterified μmoles	P:O
Succinate	None	7.06	6.86	0.97
	Frozen	6.07	3.16	0.52
	Frozen and Heated	6.87	5.86	0.85
	Heated	6.78	8.00	1.17
	Heated and Frozen	5.71	5.00	0.88
	Heated-Frozen-Heated	6.16	6.86	1.11
Generated NADH	None	7.67	6.00	0.78
	Frozen	5.00	2.66	0.53
	Frozen and Heated	4.55	5.20	1.14
	Heated	6.69	7.36	1.10
	Heated and Frozen	3.92	2.80	0.71
	Heated-Frozen-Heated	3.92	4.66	1.18

Conditions were given under Figure 1. ETP were frozen at -75°C (dry ice in acetone) for 10 min.

level of phosphorylation increased (37% over the heated and frozen ETP) on reheating (Table 1). When generated NADH was used as substrate ETP first subjected to heat-treatment followed by freezing exhibited a decrease in oxidation and phosphorylation (41% and 62% respectively). Further treatment of these particles with heat resulted in an increase in the level of phosphorylation (64% over the heated and frozen ETP), while the oxidation was not changed. Similar changes though less pronounced were observed when succinate was used as substrate.

Both oxidation and phosphorylation were lost when the ETP were subjected to freezing and thawing procedure several times (Figure 2). While the phosphorylation in ETP frozen once could be reactivated by heat, the

TABLE 2

Effect of Freezing ETP in Presence of Glycerol and DMSO on Oxidative Phosphorylation

Treatment	Protective agent	Oxygen uptake μatoms	Pi esterified μmoles	P:O
None	None	6.96	6.93	0.99
Frozen	None	7.05	4.26	0.60
Frozen-Heated	None	6.16	6.40	1.03
Heated	None	6.25	8.40	1.34
None	Glycerol 20%	6.51	5.60	0.86
Frozen	Glycerol 20%	5.98	5.86	0.97
Frozen-Heated	Glycerol 20%	5.53	7.06	1.27
Heated	Glycerol 20%	5.98	6.66	1.11
None	DMSO 10%	6.33	6.13	0.96
Frozen	DMSO 10%	5.35	4.66	0.87
Frozen-Heated	DMSO 10%	5.62	6.13	1.09
Heated	DMSO 10%	4.10	5.46	1.33

Conditions have been described under Figure 1. Generated NADH was used as the substrate. Glycerol (20%) or DMSO (10%) were used during freezing (-75°, 10 min) or heating.

response was very poor in the ETP frozen twice. In the ETP frozen five times, there was a considerable loss of both oxidation and phosphorylation and these particles did not respond to subsequent heating.

Respiratory control has been used as an index of the structural integrity of mammalian mitochondria. This activity has not been demonstrated in the M. phlei system. Respiratory control was lost when mitochondria were frozen (15, 16, 17, 18). However, mitochondria could be protected from the effects of freezing by cryoprotective agents such as glycerol (11, 12) and dimethyl sulfoxide (DMSO) (11, 14). Protection from freezing was observed with M. phlei ETP in the presence of glycerol (20%) and DMSO (10%) (Table 2). It was also observed that a mixture of Mg^{++} and ATP and soluble coupling factors protected almost completely from the effects of freezing (results not shown in the table).

The interesting feature arising out of the present studies is that heat-treatment could reverse the effects of freezing and thawing on oxidative phosphorylation in the M. phlei system. However, such a phenomenon could not be demonstrated in rat liver mitochondria or submitochondrial particles. Since freezing and thawing is known to alter the membrane structure, it may suggest that these reversible changes brought about by heat-treatment of the M. phlei ETP represent alterations in the structural organization of the membrane vessicles. Structural integrity has also been shown to be critical for succinate oxidase activity and for phosphorylation (14) both of which increased by heat-treatment in M. phlei system (1).

ACKNOWLEDGMENTS: This work was supported by grants from NIH, USPHS (AI 05637), the NSF (GB 6257X) and by the Hastings Foundation of the University of Southern California School of Medicine. This is the 54th paper in a series dealing on oxidative phosphorylation in fractionated bacterial systems. We wish to acknowledge the technical assistance of Miss Patricia Brodle and Mrs. Keiko Kikekawa.

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