THE EFFECT OF TRYPSIN AND HEAT TREATMENT ON OXIDATIVE PHOSPHORYLATION IN MYCOBACTERIUM PHLEI

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SUMMARY: The electron transport particles from Mycobacterium phlei contain particle-bound coupling factors and require the addition of soluble coupling factors for restoration of phosphorylation. However, exposure of the particles to heat (50°) or treatment with trypsin increased the P/O ratio with succinate or with NAD^+-linked substrates. In addition, the heat treated particles no longer required the soluble coupling factors for phosphorylation, but the trypsin-treated particles required this addition for maximal levels of phosphorylation. A combination of treatment with trypsin and heat resulted in higher levels of phosphorylation than either treatment alone. The phosphorylation observed in both cases was sensitive to uncoupling agents.

INTRODUCTION: Oxidative phosphorylation in bacterial systems generally exhibits low P/O ratios; in part this appears to be due to the presence of nonphosphorylative (cyanide insensitive) NADH oxidases which results in the bypass of electrons from the major respiratory chain (1). Although the P/O ratios were higher with fractionated bacterial systems and were increased by the addition of soluble coupling factors to these electron transport particles (ETP) (2). The P/O ratios were still lower than observed with mammalian mitochondria, even with the bacterial systems in which three phosphorylative sites are operative (3). The role of the soluble coupling factors is not clear; however, it is of interest that following heat treatment of the ETP (50°) for 15 min., the soluble coupling factors are no longer required (4). This finding may indicate that the soluble factors act by removing a natural inhibitor or regulator present in the ETP.

Treatment of the ETP with trypsin was found to result in an increase in the level of phosphorylation. Trypsin treatment of ETP of M. phlei has been shown to

result in the unmasking of latent ATPase (5). In contrast, heat treatment failed to unmask latent ATPase.

It is the purpose of this communication to demonstrate that treatment of the ETP of M. phlei with pancreatic trypsin or heat or both treatments results in particles which appear to be more coupled. In addition, some of the properties of both types of treated particles are compared.

METHODS AND MATERIALS: Mycobacterium phlei ATCC 354, was grown and harvested according to methods previously described (6). The ETP and the supernatant containing the coupling factors were prepared as described by Brodie (2). The ETP were washed with 0.15 M KCl containing 0.01 M $MgCl_2$ and Tris HCl buffer (0.01 M), pH 7.4 and centrifuged at 144,000 x g for 60 min. The crude supernatant was treated with ammonium sulfate and the fraction which precipitated between 35-65% saturation was collected, dissolved in 0.001 M Tris-HCl buffer, pH 7.4 and dialyzed against 0.001 M Tris HCl buffer (pH 8.0) for 48 hrs.

Oxidation was measured manometrically with a Gilson differential respirometer or polarographically with a Yellow Springs Oxygen Monitor at 30°. Phosphorylation was measured by the disappearance of orthophosphate from the reaction mixture as determined by the method of Fiske and SubbaRow (7).

Whole cells or the ETP were treated by heating at 50° for 10-15 min. The ETP were treated with trypsin (1.0 mg trypsin per 20 mog of ETP protein) for 10 min. at 30°. The reaction was terminated by the addition of trypsin inhibitor (2.0 mg of inhibitor per 1.0 mg of trypsin).

ATPase activity was measured by the release of orthophosphate from ATP. The reaction was carried out for 15 min. at 30°C. The reaction was terminated by the addition of 1.0 ml trichloroacetic acid (10%). Inorganic orthophosphate released from ATP was measured by the procedures described above (7). Protein concentration was determined by the turbidimetric method of Stadtman et al (8).

RESULTS AND DISCUSSION: Low levels of phosphorylation occur with the ETP of bacterial origin with added NADH as the substrate, (Table 1). Heating of the

TABLE 1

Effect of Heating Electron Transport Particles on Oxidative Phosphorylation

Substrate	Before Heating			After Heating		
	O_2 μatoms	Pi μmoles	P/O	O_2 μatoms	Pi μmoles	P/O
NADH	9.8	2.3	0.23	8.3	6.1	0.73
NADH + gramicidin A	10.6	0.0	0.0	10.4	0.0	0.0
NADH + KCN (5×10^{-3}M)	1.8	0.0	0.0	1.3	0.0	0.0
Ethanol	9.6	6.1	0.63	8.6	8.4	0.98
Ethanol + gramicidin A	10.8	0.0	0.0	10.3	0.0	0.0

The reaction mixture consisted of M. phlei particles (2.0 mg protein), 100 μmoles HEPES-KOH buffer pH 7.4, 50 μmoles glucose, 15 μmoles orthophosphate, 15 μmoles $MgCl_2$, 3.0 mg yeast hexokinase, 5 μmoles ATP, gramicidin A (2 μg/ml protein) where indicated, and water to a final volume of 2.0 ml. The reaction was started by the addition of 15 μmoles of NADH or 100 μmoles ethanol from the side arm. With ethanol as the substrate the reaction mixture also contained 25 μmoles hydrazine, 0.5 mg alcohol dehydrogenase and 1.0 μmole NAD^+. The reaction was run for 10 min. at 30°C, and was stopped by the addition of 1.0 ml 10% trichloroacetic acid. Following centrifugation an aliquot of the supernatant was used for phosphate determination.

ETP at 50°C for 15 min., resulted in lower level of oxidation, (11%); however, of particular interest was the finding that following heat treatment the level of phosphorylation increased about 3 fold over the control nontreated system (Table 1). Higher levels of phosphorylation were obtained with generated NADH, as well as with added NADH. Similar results were also obtained with succinate as the electron donor (4). The increased phosphorylation following heat treatment was sensitive to gramicidin A (2μg per milligram of protein). Furthermore, there was a stimulation of oxidation in the presence of gramicidin A (Table 1).

The mechanism of activation of the coupled pathway by heat is not known. There are indications that a heat sensitive inhibitor may be bound to the ETP. This inhibition may be neutralized or inactivated by the addition of the soluble "coupling" factors; however, following heat treatment of the ETP the soluble "coupling" factors are not required for phosphorylation (4).

Another possible explanation for the increased level of phosphorylation with treated particles was that heat treatment destroyed the nonphosphorylative (cyanide insensitive) NADH oxidase which bypasses the phosphorylative pathway (3). However, no significant differences were observed in the bypass reactions follow-

ing heat treatment. The inhibition of oxidation by KCN was 82 to 85% with the regular or heat treated particles respectively (Table 1). In addition, as seen in Table 1 there is an absolute increase in the level of phosphorylation (3 times) and slight inhibition of oxidation.

Trypsin treatment of the ETP of M. phlei was found to result in an unmasking of latent ATPase (5), similar to that described for mammalian mitochondria (9). Thus it was of interest to compare the effect of heating with that of trypsin on the ETP since the latter treatment unmasked the latent ATPase associated with the particulate bound coupling factor. Trypsin treatment of the ETP was found to result in lower level of oxidation (15%) and in a higher level of phosphorylation than that observed with untreated particles (Table 2). The increased level of phosphorylation was found to be sensitive to gramicidin A and KCN; however, the level of cyanide sensitivity was increased about 9 to 11%. Nevertheless, this does not appear to be the explanation for the increased level of phosphorylation since the latter activity was increased about 60%. An additional difference between the heat and trypsin treated particles was found in their requirement for soluble "coupling" factors (AS II) since trypsin treated particles require the addition of AS II for maximal levels of phosphorylation whereas the addition of AS II to the heat treated particles failed to stimulate the level of phosphorylation. It is also of interest to note that trypsin treatment of

TABLE 2

The Effect of Trypsin Treatment and Heating of the Electron Transport Particles on the Oxidative Phosphorylation

Addition	ETP			+ Trypsin Treated			+ Heat		
	O_2 μatoms	Pi μmoles	P/O	O_2 μatoms	Pi μmoles	P/O	O_2 μatoms	Pi μmoles	P/O
None	9.2	2.5	0.27	7.9	4.0	0.51	8.6	5.1	0.59
AS II	9.4	5.3	0.56	8.4	6.3	0.75	8.8	5.5	0.66
KCN	1.8	0.0	0.0	0.6	0.0	0.0	1.3	0.0	0.0
gramicidin A	9.8	0.0	0.0	8.5	0.0	0.0	9.0	0.0	0.0
AS II + gramicidin A	9.9	0.0	0.0	8.7	0.0	0.0	9.2	0.0	0.0

Conditions as in Table 1. Substrate was 15 μmoles of NADH.

the ETP resulted in a loss of succinoxidase activity (60-80%) whereas following heat treatment succinoxidase activity is increased 20 to 50% (4).

Particles treated with trypsin and heat were compared to untreated particles (Table 3). Higher levels of phosphorylation were observed with particles following both types of treatment than those obtained with particles treated with trypsin or heat alone (see Table 2). It appears that the increased level of phosphorylation following combined treatment of the ETP resulted in an additive increase in the level of phosphorylation. The soluble coupling factors were not required for maximal phosphorylation following combined treatment of the ETP. The phosphorylation following both types of treatment was sensitive to gramicidin A. No significant difference in activity was found on reversing the order of treatment.

TABLE 3

The Effect of a Combination of Trypsin Treatment and Heating of ETP on Oxidative Phosphorylation

Addition	Before Treatment			After Treatment		
	O_2	Pi	P/O	O_2	Pi	P/O
None	11.8	4.6	0.39	7.1	10.8	1.52
gramicidin A	12.6	0.0	0.0	10.5	0.0	0.0

The conditions were similar to those described in Table 1. Ethanol was used as the substrate. The ETP were treated with trypsin for 10 min. at 30°, trypsin inhibitor added and the particles heated at 50° for 15 min. The amount of trypsin and trypsin inhibitor used is described under methods.

TABLE 4

The Effect of Heat and Trypsin Treatment of the ETP on Latent ATPase

Treatment	ATPase Activity mμmoles Pi released/min/mg protein
None	15
Trypsin	57
Trypsin; Heat	90
Heat	18
Heat; Trypsin	75

The reaction mixture consisted of ETP (3.0 mg protein), 50 μmoles HEPES-KOH buffer pH 7.4, 3.0 μmoles $MgCl_2$ and water to a final volume of 1.0 ml. The reaction was started by the addition of 10 μmoles of ATP, and was run at 30°C for 15 min. The reaction was stopped by the addition of 1.0 ml 10% trichloroacetic acid.

The optimal level of phosphorylation was observed following 10 min. of treatment with pancreatic trypsin (1 mg trypsin per 20 mg protein) and 15 min. of heat treatment at 50°. Longer incubation of the ETP with trypsin was found to result in the destruction of their ability to carry out oxidation and phosphorylation.

A further indication that trypsin treatment differed in its effect from heating was found in the unmasking of ATPase (Table 4). Untreated ETP exhibit low activity of ATPase. Following trypsin treatment, the activity was increased almost 400%, whereas following heat treatment latent ATPase was not unmasked. Heat treatment following trypsin treatment increased the ATPase activity to its highest level. Heating the ETP prior to trypsin treatment was found to result in a higher level of ATPase activity than trypsin treatment alone, but not as much as that obtained following trypsin treatment prior to heating (Table 4).

In conclusion, trypsin or heat treatment of the ETP was found to result in low levels of oxidation but increased levels of phosphorylation. The nature of these effects is not clear at present; however, the two types of treatment differ. Trypsin treatment has been shown to effect partially the cyanide insensitive non-phosphorylative electron transport pathway and to unmask the latent ATPase associated with the particle-bound coupling factor (6). In addition following trypsin treatment of the ETP maximal levels of phosphorylation require the addition of the soluble coupling factors. In contrast, heat treatment of the ETP failed to unmask the latent ATPase associated with the particle and does not require the addition of soluble coupling factors for maximal levels of phosphorylation. It would appear that heat treatment either results in a structural change or acts by removing a natural inhibitor or regulator of the coupling events. Stimulation by heat treatment of the ETP has also been observed with Micrococcus lysodeikticus (10); however, this effect was not observed with beef heart mitochondria or submitochondrial particles.

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