ENERGY DEPENDENT STRUCTURAL CHANGES IN THE MEMBRANES OF M. PHLEI

V.K. Kalra, H. N. Aithal and A.F. Brodie

Department of Biochemistry
University of Southern California School of Medicine
Los Angeles, California 90033

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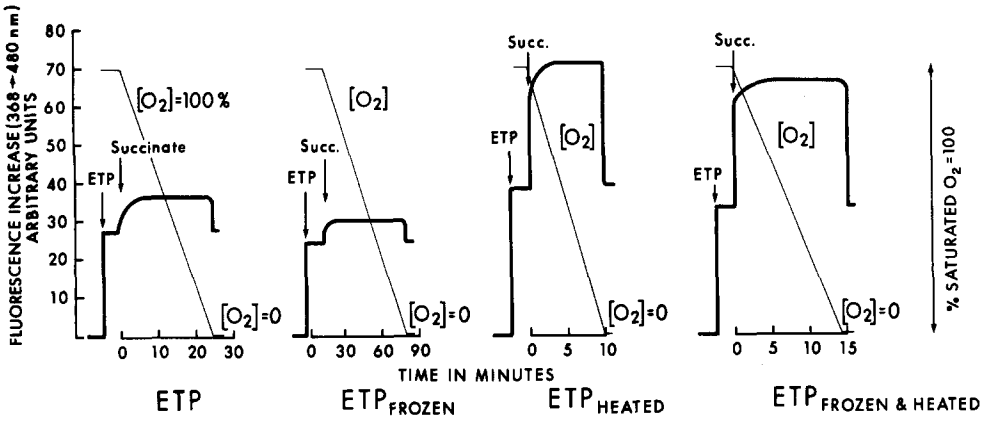
SUMMARY: The system from Mycobacterium phlei exhibited both nonenergized and energized fluorescence similar to that observed in mammalian mitochondrial systems. Conditions which affect membrane structure were found to influence both the level of oxidative phosphorylation and the energized state. However, a direct correlation between the level of phosphorylation and the energized state was not observed. Heat treatment of the membranes resulted in an enhancement of the nonenergized and energized fluorescence, whereas a decrease in fluorescence was observed following freezing. There was a significant decrease in the dissociation constant of the heat-treated membrane-dye complex with no change in the number of binding sites suggesting a change in the membrane structure.

Oxidative phosphorylation in bacterial systems is dependent on the structural integrity of a highly organized particulate fraction, the electron transport particles (ETP) (1-6). The ETP from M. phlei exhibited an interesting phenomenon of increased phosphorylation when subjected to heat treatment (50° for 10 min) which eliminated the requirement for soluble coupling factors for maximal activity (7). In contrast, freezing the ETP resulted in a decreased phosphorylation which could be reactivated by a subsequent heat treatment (8). This suggested an alteration in the structure of membrane induced by different treatments leading to altered phosphorylative capacity. The fluorescent dye, 1-anilino-8-naphthalene sulfonate (ANS) has been extensively used to detect conformational changes in proteins and membranes (9-17). Submitochondrial particles have been shown to elicit ANS fluorescence which increased on energization by the addition of ATP or oxidizable substrates such as succinate (9, 10, 16, 17). This increase has been attributed to changes in the membrane structure (9, 18). However, similar results in the bacterial systems have

been lacking (18). Hence, studies using ANS-probe have been undertaken to explain the possible structural changes induced in the ETP from M. phlei by heating and freezing and the results are reported in this communication.

MATERIALS AND METHODS: The methods for preparation of ETP from M. phlei and the measurement of oxidation and phosphorylation have been described previously (6, 19). ANS fluorescence was measured with a Baird-Atomic Fluorispec SF-100, connected to a multispeed recorder, at 480 nm with an excitation wavelength of 368 nm. The increase in fluorescence on the addition of ETP to a medium containing ANS and the further increase obtained on addition of succinate are referred to as nonenergized (NE_f) and energized (E_f) fluorescences respectively. ANS binding was measured after incubation of 20 mg of ETP with 5 μmoles of ANS at room temperature for 5 min in presence of 250 μmoles of tris-acetate buffer (pH 7.2) and 100 μmoles of Mg^{++} in a total volume of 3.0 ml. Subsequently the samples were rapidly cooled to 0° and centrifuged at 144,000 x g for 40 min. The amount of dye in the supernatant (unbound) and in the pellet (bound) were determined in the presence of saturating amount of bovine serum albumin (20). The dissociation constant (K_D) were calculated according to the method of Cheung and Morales (21). Protein was determined by biuret method (22) and inorganic phosphate was determined by the method of Fiske and SubbaRow (23).

RESULTS AND DISCUSSION: When ETP were added to a medium containing ANS, there was an increased fluorescence (NE_f) (Fig. 1). Addition of succinate increased the fluorescence (E_f) further which disappeared when the suspension became anaerobic (Fig. 1) or when inhibitors of oxidation or uncoupling agents were added (results not shown in the figure). In the heat-treated ETP the levels of NE_f and E_f increased by 11 and 24 units respectively compared to untreated ETP. The increase in P/O ratio was only 40% in the heat-treated preparations due to increased phosphorylation (Table 1). In contrast, frozen and thawed ETP exhibited a decreased E_f (3 units) compared to untreated ETP though the P/O ratio decreased by 22%. It is of interest that when the frozen and thawed ETP were heated the NE_f and E_f almost increased to the same level of heat-



Anilino-naphthalene sulfonate response of ETP under various conditions.

The reaction mixture contained in a final volume of 3 ml, 50 mM Tris-acetate, pH 7.2, 70 μM anilino-naphthalene sulfonate (ANS). Additions of particles (ETP, 0.72 mg protein) and 20 mM succinate were made at times indicated. ETP(frozen) were prepared by freezing ETP at -75° for 10 min, followed by slow thawing; ETP (heated) were prepared by heating at 50° for 10 min; ETP (frozen and heated) were prepared by freezing ETP to -75° for 10 min, slow thawing, and then heating at 50° for 10 min. The excitation wavelength was 368 nm and the emission was 480 nm. Oxygen uptake was followed using a vibrating platinum electrode.

treated ETP, although P/O ratio increased to the level of untreated ETP. Thus, a direct correlation between changes in E_f and phosphorylation could not be established; yet changes in both reflect change in structure of membrane.

A comparison of the periods required for the different ETP preparations to reach anaerobiosis also revealed significant differences (Fig. 1). Whereas untreated ETP required 25 min to reach anaerobiosis, heat-treated ETP required only 10 min while frozen ETP required 80 min. Heating the frozen ETP reversed the effect of freezing and resulted in shortening the time required for anaerobiosis to 15 min which was almost the same as that required by heat-treated ETP.

It was of interest to determine whether the increase in the E_f (about 3-fold) in the heat-treated ETP reflected a change in the number of binding sites or a change in the apparent dissociation constant (K_D) of the dye-ETP complex. There was an increased number of binding sites when the different ETP preparations were energized (Table 1). However, no difference was

Effect of different treatment on oxidative phosphorylation and ANS response in energized and nonenergized membranes of electron transport particles of M. phlei

	Oxygen uptake (μatoms)	Pi esterified (μmoles)	P/O	Increase in fluorescence (arbitrary units)		Binding sites (nanomoles/mg prot)		Dissociation constant (K_D) (x 10^{-5}M)	
				NE_f	E_f	NE_f	E_f	NE_f	E_f
ETP	5.80	6.53	1.12	27	9	23	44	16.7	10.0
Frozen ETP	5.53	4.82	0.87	24	6	22	37	17.0	12.5
Heated ETP	6.78	10.66	1.57	38	33	26	46	15.0	2.7
Frozen and Heated ETP	6.71	6.80	1.19	33	31	25	44	15.5	4.5

The reaction mixture for oxidative phosphorylation contained ETP (5.0 mg protein), 100 μmoles of HEPES-KOH buffer, pH 7.4, 50 μmoles glucose, 15 μmoles orthophosphate, 30 μmoles $MgCl_2$, 3.0 mg hexokinase, 2.5 μmoles AMP, 25 μmoles KF and 50 μmoles of succinate in a final volume of 2.0 ml. The oxygen uptake was measured for 15 min at 30° using a Gilson differential respirometer and the reaction was stopped by the addition of 1ml of 10% TCA. The samples were centrifuged and aliquots of supernatant was used for phosphate determination by the method of Fiske and SubbaRow (23). Heat treatment was at 50° for 10 min. Freezing was carried out at -75° for 10 min followed by slow thawing (8). The binding studies were carried out using 20 mg of various ETP preparations as described under Materials and Methods. Energization was brought about by 250 μmoles of succinate. The recovery of added ANS in these experiments were between 92-96%.

observed in the binding sites in the energized states of different preparations. Untreated or heated ETP when incubated with ANS and washed free of unbound ANS, still exhibited both NE_f and E_f without further addition of ANS similar to changes observed in the unwashed preparations. Thus during the transition to the energized state, the bound ANS molecules migrated toward a more hydrophobic environment as a result of some conformational change.

A comparison of dissociation constant of the different ETP-dye complex revealed a significant decrease in the heat-treated ETP, about 3.5 times less compared to the untreated ones. In the frozen ETP this value increased by about 1.25 times which decreased on subsequent heating (Table 1). Thus, these results reflect a change in the membrane structure. However, changes in the K_D values might reflect a change in the membrane charge as has been suggested for the mitochondrial system (18). Membrane potential have been implicated in the changes in ANS-fluorescences in mitochondrial systems under nonenergized and energized states (20). A charge separation in a special locus or region of the membrane distinct from the nonenergized site have thus been implicated in the energy dependent ANS fluorescence (17). However, it is possible that enhanced fluorescence may be brought about by a change in membrane potential accompanying a change in membrane structure.

The present data suggest a structural alterations in the membranes of ETP subjected to different treatments. It is interesting that the alteration brought about by freezing was reversed by heating. Although the structural alterations influence both the levels of phosphorylation and ANS-fluorescences, the direct relationship between the two is not clear.

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