STUDIES OF ENERGY LINKED REACTIONS: A ROLE FOR LIPOIC ACID IN THE PURPLE MEMBRANE OF *HALOBACTERIUM HALOBIUM*

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1. Introduction

The bacteriorhodopsin system of *Halobacterium halobium* has been widely investigated [1,2] as a light driven system for generation of an electrochemical potential of H^+ ions ($\Delta\bar{\mu}H$) which is utilised for ion transport [3] and coupled ATP synthesis [4]. The bacteriorhodopsin is localised in a membrane region (purple membrane) which contains no other proteins but contains 25% lipid by weight and whose only known function is that of a light-driven proton pump. It is claimed that light generated $\Delta\bar{\mu}H$ is utilised to drive a proton translocating ATP synthase which is located spatially in a different region of the plasma membrane [5]. The postulated mechanism of ATP synthesis has thus provided major experimental support for the chemiosmotic hypothesis [6]. The demonstration of light-driven uncoupler-sensitive proton movements [7], light-generated membrane potential and DCCD sensitive photophosphorylation have all been taken as evidence in support of chemiosmotic coupling [8] and Skulachev [9] suggests that the link between purple membrane and ATP synthase containing membrane region occurs via $\Delta\bar{\mu}H$ transfer along the membrane over large distances. This chemiosmotic interpretation has received further support from the experiments of Racker and Stoeckenius [4] who demonstrated light-dependent ADP phosphorylation in proteoliposomes made of soya-bean phospholipids, purple membrane and an ox heart ATP synthase complex made of hydrophobic protein [10] and F_1 ATPase. It is assumed that there is no functional link between the purple membrane and the components of the reconstituted ATP synthase complex other than a light-generated proton gradient.

Current studies in this laboratory have indicated a role for lipoic acid residues in oxidative phosphorylation and that lipoate is involved, with *cis* Δ9 monoenoic acids such as oleic acid, in a cycle of reactions analogous to those involved in substrate level phosphorylation [11]. It is proposed that lipoate residues in the mitochondrial inner membrane act as a mobile pool of cofactors which provide a link between the respiratory chain and the ATP synthase complex [11].

This paper demonstrates a similar link role for lipoic acid in photophosphorylation reactions catalysed by the purple membrane from *Halobacterium halobium* in a system analogous to that described by Racker and Stoeckenius [4]. In addition, it is shown that:

(i) Purple membrane contains large amounts of lipoic acid indicating a specific cofactor (or electron acceptor) function for lipoate.

(ii) That purple membrane catalyses the rapid photoreduction of lipoate.

(iii) That the photophosphorylation reaction involves direct transfer of dihydrolipoate from the purple membrane system to mitochondrial membranes or *E. coli* vesicles.

2. Materials and methods

The growth of *Halobacterium* and isolation of purple membrane was as described by Oesterhelt and Stoeckenius [12]. The purple membrane was purified by density-gradient centrifugation and washed in a centrifugation buffer of 0.25 M sucrose, 10 mM Tris–

Cl, 1 mM EDTA, at pH 7.5. In some experiments the purple membrane was also washed by passage twice through a column of Sephadex G-25. The protein composition of the purple membrane was estimated by Folin reagent and also from the extinction coefficient for bacteriorhodopsin [13]. Ox heart submitochondrial particles (SMP), ATP synthase preparations such as Complex V [14] and membrane vesicles from lipoate-deficient *E. coli* mutant W1585 lip 2 [15] were prepared as described previously [11,16]. Assays for ATP synthesis in a glucose–hexokinase trap system, and assays for inorganic phosphate, glucose-6-phosphate and mitochondrial protein were as described previously [11] as were the sources of chemicals, inhibitors and enzymes used.

Photoreduction of exogenous lipoate by purple membrane in a 0.25 M sucrose, 10 mM Tris–Cl, 1 mM EDTA medium, at pH 7.5, was carried out by exposure to laboratory fluorescent lighting. Samples were taken at 20 s intervals for dithiol assay, using the Ellman reagent [11].

Photophosphorylation experiments using a glucose–hexokinase trap medium were carried out by simply mixing purple membrane with SMP or Complex V by stirring in an open cuvette and irradiating with a 500 W projector fitted with a cut-off filter transmitting maximally between 500–600 nm. Samples were taken for assay of inorganic phosphate uptake and glucose-6-phosphate formation. Photophosphorylation experiments were also carried out in a Perspex dialysis chamber in wich the two halves of the chamber were separated by dialysis tubing (Spectrophore, cut off 3000 mol. wt). Both chambers contained the glucose–hexokinase trap phosphorylation medium [11] but purple membrane was added to one chamber and SMP to the other chamber. Both chambers were stirred with magnetic stirrer 'fleas' and after incubation in the light or the dark, both chambers were sampled for inorganic phosphate disappearance or glucose-6-phosphate formation.

Lipoate content of purple membrane was assayed by bioassay as described by Herbert and Guest [15] except that no acid hydrolysis treatment was necessary with purple membrane, indicating that lipoate was present in a 'free' or readily available form. Lipoate content was also assayed by titration with dibutylchloro-[^{3}H]methyltin chloride as described for mitochondrial membranes [17,18].

3. Results

The demonstration of dihydrolipoate-dependent ATP synthesis catalysed by submitochondrial particles and Complex V [11] suggested another interpretation of the mechanism of light-dependent ATP synthesis in proteoliposomes containing purple membrane and heart mitochondrial membrane fraction [4] and the photoreduction by purple membrane of lipoate components of the mitochondrial inner membrane was investigated. Table 1 shows net ATP synthesis achieved by simply irradiating a mixture of purple membrane (PM) and submitochondrial particles (SMP) in a glucose–hexokinase trap system. Addition of lipoate leads to an enhancement of ATP synthesis. ATP synthesis is sensitive to inhibitors such as oligomycin and DCCD and to uncoupling agents such as FCCP and '1799' but is insensitive to TTFB and to ionophores such as valinomycin and nigericin. This inhibitor sensitivity is similar to that which is seen in studies of dihydrolipoate-dependent ATP synthesis in all systems examined ([11], Griffiths, D. E. and Hyams, R. L. unpublished work).

Table 2 shows the results of experiments with mixtures of purple membrane and a purified ATP synthase preparation, Complex V from ox heart mitochondria. For ATP synthesis an additional cofactor is required, a *cis* Δ9 monoenoic acid such as oleate, as was found in previous studies of dihydrolipoate-dependent ATP synthesis by purified ATP synthase preparations [11,19]. Addition of lipoate again leads to an enhancement of ATP synthesis. There is no requirement for oleoyl CoA in these experiments in contrast to previous studies [11] of dihydrolipoate-dependent ATP synthesis by Complex V. However, addition of oleoyl CoA stimulates ATP synthesis and there is a further enhancement on addition of lipoate. These experiments together with those described in table 1 indicate that purple membrane is catalysing photoreduction or reductive acylation of a component of the mitochondrial membrane and of Complex V, and that this component is either lipoate or a component which reduces lipoate.

The following observations support this conclusion:

(i) Purple membrane catalyses a very rapid photo-

Table 1
Photophosphorylation of ADP by a mixture of purple membrane (PM) and submitochondrial particles (SMP)

Additions to incubation medium	Light/Dark	ΔP_i (μmol/20 min)
Experiment A		
1. SMP	L or D	0.0
2. PM	L or D	0.0
3. SMP + Dihydrolipoate (1 μmol)	D	1.14
4. PM + Dihydrolipoate (1 μmol)	L or D	0.0
5. SMP + PM	D	0.0
6. SMP + PM	L	0.69
7. SMP + PM + lipoate (1 μmol)	D	0.0
8. SMP + PM + lipoate (1 μmol)	L	1.79
9. SMP + lipoate (1 μmol)	L	0.0
Experiment B		
1. SMP + PM	L	1.07
2. SMP + PM + FCCP[a] (μg)	L	0.0
3. SMP + PM + '1799'[a] (10 μg)	L	0.0
4. SMP + PM + TTFB[a] (10 μg)	L	1.05
5. SMP + PM + valinomycin (10 μg)	L	1.04
6. SMP + PM + nigericin (10 μg)	L	0.91
7. SMP + PM + valinomycin (10 μg) + nigericin (10 μg)	L	1.11
8. SMP + PM + oligomycin (10 μg)	L	0.0
9. SMP + PM + lipoate (1 μmol)	L	1.75

[a]FCCP, *p*-trifluoromethyloxy carbonyl cyanide phenylhydrazone
1799, bis-hexafluoro acetonyl acetone
TTFB, 4,5,6,7-tetrachloro-2-trifluoromethyl-benzimidazole

ATP phosphorylation was assayed in a glucose–hexokinase trap system as previously described. The basic reaction mixture contained 250 mM sucrose, 22 mM glucose, 2 mM $MgCl_2$, 0.2 mM EDTA, 2 mM ADP, 4.2 mM potassium phosphate, 20 mM Tris–Cl, pH 7.3 and 25 units hexokinase. Submitochondrial particles (1 mg protein) inhibited with antimycin A (5 μg) and rotenone (5 μg) were added and the reaction was started immediately by the addition of 0.4 mg purple membrane. The reaction mixture was irradiated with a 500 W projector lamp fitted with a cut off filter transmitting maximally between 500–600 nm. Total vol. 1.1 ml; Temp. 30°C. Samples were taken after 20 min for inorganic phosphate disappearance or glucose-6-phosphate formation. Phosphate disappearance was shown to correlate with glucose-6-phosphate formation in all cases so only the values for phosphate disappearance are shown.

Table 2
Light-dependent ATP synthesis by mixtures of purple membrane (PM) and Complex V (CV)

Additions to medium		Light/Dark	ΔP_i (μmol/20 min)
1.	CV + PM	D	0.0
2.	CV + PM	L	0.0
3.	CV + PM + lipoate	L	0.0[a]
4.	CV + PM + oleate	L	0.52
5.	CV + PM + oleate + oleoyl CoA	L	0.94
6.	CV + PM + oleate + lipoate	L	1.20
7.	CV + PM + oleate + oleoyl CoA + lipoate	L	1.97[b]
8.	Same as 7 + oligomycin (10 μg)	L	0.0

[a]With some preparations of Complex V a low rate of phosphorylation was observed
[b]This reaction is inhibited by '1799' (10 μg), FCCP (10 μg) and DCCD (10 μg). Also, no phosphorylation was observed in incubation in the dark

The incubation medium for ATP synthesis with Complex V was as described previously [11] and the experimental conditions are described in table 1. Additions to the medium were Complex V (0.5 mg protein), oleate 10 nmol, oleoyl CoA 10 nmol, lipoate 1 μmol. The reaction was started by the addition of purple membrane (0.4 mg protein). Incubation with stirring for 20 min at 30°C.

reduction of added lipoate. Minimal estimates of the rate of photoreduction give values of 17–20 nmol lipoate reduced/nmol bacteriorhodopsin/s.

(ii) Purple membrane preparations contain very large amounts of lipoic acid (100–150 nmol lipoate/mg protein, i.e. 3–4 mol lipoate/mol bacteriorhodopsin) when assayed by dibutylchloro-[^{3}H] methyltin chloride binding [17,18] and by lipoate bioassay [15]. This lipoate is 'free' or readily available for bioassay as no prior acid hydrolysis or solvent extraction is required as in the case of mitochondrial and chloroplast membrane preparations.

These findings indicate that a pool of lipoate cofactors provides the link between the photoreductive proton-translocating purple membrane system and the ATP synthase complex as previously postulated for respiratory chain oxidative phosphorylation [11,19].

Evidence for a mobile diffusible cofactor and for direct transfer of lipoate residues from purple membrane to membrane-bound ATP synthase is presented in tables 3 and 4. Light-dependent phosphorylation is still achieved when the two catalytic components are separated by a semi-permeable dialysis membrane (table 3) and the results are explainable by net transfer by diffusion of dihydrolipoate, a product of photoreduction by purple membrane, which interacts with submitochondrial particles to generate ATP as previously described [11]. Table 4 summarises an experiment in which oxidative phosphorylation with succinate as substrate is restored to lipoate deficient vesicles from a lip$^-$ mutant of *E. coli* by addition of purple membrane in the dark. Restoration of oxidative phosphorylation by addition of lipoate to lipoate deficient vesicles has been described previously [16] and the degree of restoration of oxidative phosphorylation is consistent with the amount of lipoate known to be present in the purple membrane.

4. Discussion

The demonstration that the purple membrane of *Halobacterium* contains a photoreducible cofactor, lipoic acid, provides a simple chemical explanation as to how the photochemical reaction in the purple membrane is linked to the membrane-bound ATP synthase complex and provides a reaction mechanism

Table 3
Evidence for a diffusable cofactor in photophosphorylation catalysed by purple membrane and sub-mitochondrial particles

Sample from:	Light/Dark	ΔP_i (μmol/20 min)	ΔG-6-P (μmol/20 min)
SMP + PM	D	0.0	0.0
SMP chamber	L	5.70	5.15
PM chamber	L	5.35	4.64
SMP + PM chamber (total)	L	11.05	9.79

A photophosphorylation experiment was carried out as described in table 1, except that the purple membrane was separated from submitochondrial particles by a semi-permeable membrane in a perspex dialysis vessel, as described in Materials and methods. Both chambers contained 5 ml glucose–hexokinase trap medium and were stirred with magnetic stirrer 'fleas'. Purple membrane (0.875 mg protein) was added to one chamber and SMP inhibited with antimycin A and rotenone (4 mg protein) to the other chamber. The apparatus was then illuminated as described in table 1. Incubation was for 20 min at 25°C. Samples were taken for P_i uptake and glucose-6-phosphate formation as described previously [11].

Table 4
Restoration of oxidative phosphorylation in *E. coli* vesicles by transfer of lipoate from purple membrane to lipoate-deficient membrane vesicles

Additions		Light/Dark	ΔP_i (μmol/15 min)
1.	*E. coli*	L or D	0.0
2.	*E. coli* + succinate (10 mM)	L or D	0.0
3.	*E. coli* + PM	D	0.08
4.	*E. coli* + PM	L	0.17
5.	*E. coli* + succinate + PM	D	0.38
6.	*E. coli* + succinate + PM	L	0.64

Oxidative phosphorylation with succinate as substrate catalysed by lipoate deficient vesicles from *E. coli* mutant [15] was carried out as previously described [16] utilising the same glucose–hexokinase trap reaction medium described in table 1. Incubations were carried out in the light or dark as phosphorylation is dependent on addition of lipoate or a source of lipoate such as purple membrane. Respiration rates are not listed but were as described previously [16]. Purple membrane 16 μg protein, *E. coli* vesicles, 0.5 mg protein.

which is relevant to the mechanism of photophosphorylation in *Halobacterium halobium*. The rapid photoreduction of lipoic acid catalysed by purple membrane has a sufficiently high turnover number to account for the photophosphorylation observed. It is not known at present whether the photoreduction of lipoate involves bacteriorhodopsin alone or whether another catalytic component is required. Examination of the purple membrane preparations by gel electrophoresis indicates the presence of a major component of mol. wt 25 000–26 000 and a minor component of lower molecular weight. It is not known if this minor component is a contaminant or a necessary component in the reaction sequence, e.g., an iron sulphur protein which has been shown to be present in large amounts in Halobacteria [20].

These studies provide a model system for studies of *Halobacterium* photophosphorylation and are relevant to the mechanism of mitochondrial oxidative phosphorylation and chloroplast photophosphoryla-

tion. The system described, a photoreduced dihydrolipoate-dependent ATP synthesis, is similar to dihydrolipoate-dependent ATP synthesis demonstrated in heart mitochondria, liver mitochondria, yeast mitochondria, *E. coli* vesicles, chloroplast fragments and heart, yeast and *E. coli* ATP synthase preparations ([11,15,19] Griffiths et al. unpublished work) and provides further evidence for a common role for lipoic acid in energy conserving membranes. It should be noted that the coupled photophosphorylation system is sensitive to oligomycin, DCCD and uncouplers such as FCCP and '1799'. However, the system is insensitive to uncoupling agents such as TTFB and ionophores such as valinomycin and nigericin which uncouple or inhibit oxidative phosphorylation as is found in studies of dihydrolipoate-dependent ATP synthesis (unpublished results). These systems thus differ significantly in uncoupler and ionophore sensitivity from oxidative phosphorylation and suggest that a prior energisation or activation step is missing or is not expressed in the model system or in the reaction sequence involved in dihydrolipoate-dependent ATP synthesis. In this context it should be noted that the current experiments, while indicating that purple membrane photoreduces lipoate, are not designed to detect activation of lipoate in a possible reductive acylation reaction. Experimental systems to establish or eliminate this possibility are under investigation.

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