THE RELATION OF THE LIGHT-INDUCED INCREASE IN ABSORBANCE AT 518 nm TO PHOTOPHOSPHORYLATION IN DIGITONIN SUBCHLOROPLAST PARTICLES

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1. Introduction

A proton translocating system is closely associated with energy conservation during electron transport in chloroplasts [1], mitochondria [2] and bacterial chromatophores [3]. Mitchell ([4], cf. also [5]) has pointed out that these H^+ movements might create not only a H^+ concentration gradient, but also a membrane potential across the coupling membrane, and that the sum of both represents the high energy state with drives phosphorylation. Whereas H^+ movements can be detected as a change in the pH of the suspending medium, the measurement of a membrane potential is more difficult. However, Witt and collaborators (cf. [5]) proposed that the light-induced increase in absorbance at 518 nm reflects an electric potential across the chloroplast membrane. A similar correlation has been established for the light-induced "carotenoid shift" in chromatophores [7, 8].

In subchloroplast particles prepared by sonication (SCP), photophosphorylation can occur even when the light-induced H^+ uptake is completely inhibited by NH_4Cl or amines [9]. Moreover, valinomycin enhances the uncoupling of phosphorylation in SCP by NH_4Cl [10], and ethylamine partially reverses this effect. Since the combination of valinomycin and NH_4^+ would be expected to dissipate a membrane potential, it was concluded that ethylamine, which interferes with NH_4^+ accumulation [11], may prevent the dissipation of the membrane potential.

This paper describes the following results:

1) Small subchloroplast particles (D-144 particles) prepared with digitonin [12] exhibit a light-induced H^+ uptake as well as a light-induced increase in absorbance at 518 nm.

2) These particles can synthesize ATP under conditions where the pH rise is totally inhibited; however, if both the pH rise and the absorbance increase at 518 nm are inhibited, for instance by valinomycin and NH_4Cl, phosphorylation is abolished.

3) If the uncoupling of photophosphorylation by valinomycin and NH_4Cl is reversed by adding aliphatic amines, the light-induced absorbance increase at 518 nm is restored as well.

Abbreviations:
PMS, *N*-methylphenazonium methosulfate;
CCP, carbonylcyanide *m*-chlorophenylhydrazone;
Tricine, tris(hydroxymethyl)methylglycine;
SCP, subchloroplast particles prepared by sonication;
D-144 particles, subchloroplast particles prepared with digitonin.

2. Methods

Chloroplasts were prepared from spinach leaves as previously described [13]. Subchloroplast particles (D-144 particles) were prepared with digitonin according to Anderson and Boardman [12] as described elsewhere [14].

Photophosphorylation and the assay for $^{32}P_i$ esterification were performed as described before [13]. Special conditions are given in the legends to the figure and tables. The light-induced pH rise was measured essentially as previously described [15]. The final volume was 5 ml, the temperature was 10° and 0.01 mM PMS was present instead of pyocyanine. Saturating white light was used for illumination and the cell was continuously flushed with argon to prevent oxidation of

PMS to pyocyanine. The buffering capacity of the suspending medium was kept as low as possible. The light-induced absorbance increase at 518 nm, using 540 nm as the reference wavelength, was measured in a Aminco-Chance double beam spectrophotometer modified for illumination from the side. A red filter (Corning #2403) and two heat filters were placed in the illumination light path and a blue filter (Corning #9782) was put in front of the phototube. The intensity of actinic red light (>620 nm) was about 10^5 ergs/cm^2/sec. The basic reaction mixture contained, in a volume of 3 ml, 50 mM Tricine-NaOH, pH 8.0, 50 mM NaCl or KCl, 5 mM $MgCl_2$, 3 mg of defatted bovine serum albumin and D-144 particles equivalent to 45 to 90 μg of chlorophyll. For simultaneous assay of $^{32}P_i$ esterification, the change in absorbance at 518 nm was measured in a split-beam spectrophotometer constructed by Dr. R.K. Clayton [16]. The device for illumination in this case provided a saturating intensity of red light (10^6 ergs/cm^2/sec). The same filters were used as with the double beam spectrophotometer. The absorbance at 518 nm rose rapidly on illumination and then more slowly decreased to a constant value. The differences between the absorbances of this steady state in the light and those in the dark were taken for the absorbance changes reported here.

Nigericin was graciously supplied by Dr. B.C.Pressman. Valinomycin and carbonylcyanide *m*-chlorophenylhydrazone were purchased from Calbiochem. *N*-Methylphenazonium methosulfate was obtained from Sigma. Diaminodurene was prepared from dinitrodurene by reduction with $SnCl_2$. Dinitrodurene and aliphatic amines were purchased from Aldrich Chem. Comp., and methylviologen from Mann Res. Laboratories.

3. Results

3.1. *The light-induced pH rise in D-144 particles*

In chloroplasts uncoupling and inhibition of the light-induced pH rise go in parallel. For instance nigericin plus K^+ [17] and amines inhibit phosphorylation and H^+ uptake at similar concentrations. In SCP, however, aliphatic amines totally inhibit the pH rise at concentrations which hardly affect phosphorylation [9].

D-144 particles exhibited a light-induced pH rise which was similar to that in chloroplasts [1] but smaller in extent. With 0.01 mM PMS as the mediator of electron flow, about 0.1 μequivalent of H^+ per mg chlorophyll was taken up at pH 6.5, which was 1/5 to 1/10 of the uptake in chloroplasts. At pH 6.5, 5 mM NH_4Cl inhibited the pH rise 90%, nigericin (1 μg/ml) in 50 mM KCl inhibited 100%, and 20 μM n-dodecylamine inhibited about 40%. As expected, the inhibition by amines was more pronounced at pH 8.0. At 10 μM, CCP inhibited the pH rise completely. Diaminodurene also supported H^+ uptake in D-144 particles.

3.2. *The light-induced absorbance increase at 518 nm in D-144 particles*

In the absence of a mediator of electron flow, no light-induced absorbance increase was observed [18], but with 5 mM ascorbate and 0.05 mM PMS, or with 1 mM diaminodurene a change of 0.06 OD per mg chlorophyll was recorded in the double beam spectrophotometer. This was about half the signal observed with SCP or chloroplasts. The response could be increased to 0.2 OD per mg chlorophyll at the saturating light intensity of the split beam spectrophotometer. Since the D-144 particles are small (700 Å average diameter) [14], light scattering changes did not interfere with the signal which showed a spectrum similar to that in chloroplasts [19]. The addition of 2 mM phosphate and 3 mM ADP to the reaction mixture inhibited the signal 20 to 30%. NH_4Cl at 10 mM, a concentration which abolished the pH rise but hardly affected phosphorylation, did not inhibit. The combination of valinomycin (2 μg/ml) and 2 mM NH_4Cl in NaCl medium inhibited 90% at 10^5 and about 60% at 10^6 ergs/cm^2/sec. Nigericin (1 μg/ml) in 50 mM KCl stimulated the response slightly. Valinomycin (2 μg/ml) in 50 mM KCl inhibited about 90%, whereas in 50 mM NaCl it has little effect. CCP at 10 μM also abolished the signal.

3.3. *Uncoupling of photophosphorylation in D-144 particles*

Since D-144 particles catalyse cyclic phosphorylation at the same rate as chloroplasts [14], it was of interest to test their response to several uncouplers. Cyclic phosphorylation with PMS was nearly insensitive to either NH_4Cl or nigericin and K^+ (fig. 1). Valinomycin dramatically increased the inhibition of phosphorylation by either NH_4Cl or nigericin and K^+. About ten-fold higher concentrations of NH_4Cl and valinomycin

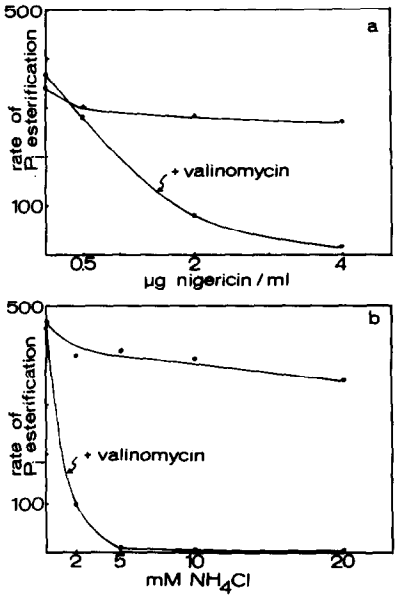

Fig. 1. Synergistic effect of uncouplers of cyclic phosphorylation in D-144 particles. PMS at 0.05 mM was used as mediator of cyclic electron flow, and particles equivalent to 17 µg chlorophyll were added to a final volume of 1 ml. Illumination was performed for 2 min with white light (2×10^6 ergs/cm^2/sec) in tubes flushed with nitrogen. The rate of P_i esterification is given in µmoles/hr/mg chlorophyll.
a) Effect of valinomycin (2 µg/ml) on the action of nigericin in 50 mM KCl.
b) Effect of valinomycin (5 µg/ml) on the action of NH_4Cl in 50 mM NaCl.

were required to yield the same degree of uncoupling in D-144 particles as in SCP [10] and the same was true for the effect of valinomycin in the presence of nigericin and K^+. At 10 µM, CCP totally inhibited phosphorylation in D-144 particles, which was the same concentration needed to inhibit phosphorylation in chloroplasts or SCP [10].

3.4. *Partial reversal of uncoupling by NH_4Cl and valinomycin on addition of aliphatic amines*

In SCP, ethylamine partially reversed the uncoupling by NH_4Cl and valinomycin [10]. More recently this effect was found for other aliphatic amines.*

In D-144 particles, only the higher homologues of the aliphatic amines yielded good reversal of the uncoupling by NH_4Cl and valinomycin. Ethylamine was inefficient up to a concentration of 50 mM. This will be discussed elsewhere*. In table 1 the effect is shown for three higher aliphatic amines. The optimal concentration of the amine needed to reverse valinomycin-induced NH_4Cl uncoupling increased with decreasing length of the hydrocarbon chain. Without NH_4Cl present, all three amines, at the higher concentrations, clearly uncoupled phosphorylation. It should be mentioned that for observation of the maximal reversal effect, the selection of the proper concentration of the amine as well as of valinomycin and NH_4Cl and of the particles was important.

To demonstrate that the stimulation of phosphorylation by aliphatic amines in the presence of NH_4Cl and valinomycin was not indirectly caused by a stimulation of electron flow, phosphorylation and electron flow were simultaneously measured. Since the D-144 particles are not able to oxidize water, photooxidation of ascorbate, mediated by diaminodurene and methylviologen, was measured polarographically [14]. It was found that oxygen uptake was unaffected by n-dodecylamine whereas phosphorylation in the presence of NH_4Cl and valinomycin was stimulated nearly threefold.

3.5. *Partial reversal of the inhibition of the light-induced absorbance at 518 nm by valinomycin and NH_4Cl by aliphatic amines, concurrent with the reversal of uncoupling*

Since the pH gradient could not be the driving force for photophosphorylation when the inhibition of phosphorylation in D-144 particles by NH_4Cl and valinomycin was reversed by aliphatic amines, we investigated the behaviour of the light-induced change in absorbance at 518 nm under those conditions. It is obvious from table 2 that the reversal of uncoupling by valinomycin and NH_4Cl by n-dodecylamine is accompanied by a restoration of the signal at 518 nm. Similar results were obtained with n-heptylamine. It has to be mentioned that the change in absorbance at 518 nm was less sensitive to valinomycin and NH_4Cl than was phosphorylation.

* R.E.McCarty, in prepartion.

Table 1
Partial reversal of uncoupling of photophosphorylation by valinomycin and NH_4Cl on adding aliphatic amines.

Additions (μM)	Rate of $^{32}P_i$ esterification (μmoles/hr/mg chlorophyll)		% of control
		+NH_4Cl	
–	268	40	14.9
n-Heptylamine			
200	195	51	26.2
500	150	72	48.0
1000	116	75	64.6
n-Nonylamine			
50	257	58	22.6
100	205	120	58.5
200	134	101	75.4
n-Dodecylamine			
5	278	94	33.8
10	238	134	54.0
20	140	76	54.3

The conditions were the same as described for fig. 1 except that 5 mM ascorbate and 2 μg valinomycin were added and illumination was performed with red light (2×10^5 ergs/cm^2/sec) under air. NH_4Cl was added to a concentration of 2 mM, where indicated.

Table 2
Partial reversal by *n*-dodecylamine of uncoupling and of concomitant inhibition of the light-induced absorbance at 518 nm by valinomycin and NH_4Cl.

Additions			Rate of $^{32}P_i$ esterification (μmoles/hr/mg chlorophyll)	ΔOD 518 nm/mg chlorophyll
n-Dodecylamine (μM)	Valinomycin (μg/ml)	NH_4Cl (mM)		
0	0	0	535	0.15
0	2	0	420	0.13
5	2	0	458	0.13
10	2	0	417	0.13
20	2	0	253	0.13
0	2	2	43	0.06
5	2	2	72	0.09
10	2	2	120	0.11
20	2	2	90	0.11

The same reaction mixture as for table 1 was used in a final volume of 2 ml. Illumination was performed in 2 periods of 30 sec in the split-beam spectrophotometer described under Methods. The changes of absorbance at 518 nm were recorded on a full chart scale of 0.055 OD. Aliquots of the reaction mixtures were assayed for $^{32}P_i$ esterification.

4. Discussion

The present experiments reveal three different types of inhibitor action on the high energy state of D-144 particles:

1) CCP totally inhibited photophosphorylation, the light-induced pH rise and the light-induced increase in absorbance at 518 nm.

2) Valinomycin and K^+ inhibited only the absorbance increase at 518 nm.

3) NH_4Cl, aliphatic amines and nigericin *plus* K^+ inhibited only the H^+ uptake.

The appropriate combination of inhibitors in the last two categories, resulted in complete uncoupling. According to Mitchell [5] synergistic action of uncouplers should occur whenever one reagent would selectively dissipate the chemical H^+ gradient and the other would facilitate the decay of the membrane potential. Synergistic effects of uncouplers, which can be explained by such a mechanism, have been reported for submitochondrial particles [20, 21], for chromatophores [22, 23] and for SCP [10]. In chloroplasts it seems that a membrane potential plays only a minor role (cf. [5]) so that synergistic effects of uncouplers should not, at first sight, be expected. Indeed it has been shown that in chloroplasts nigericin and K^+ [17] and NH_4Cl [10] completely inhibit phosphorylation without any significant additional effect of valinomycin.

If we accept that the absorbance change at 518 nm is an indicator of an electric potential across the coupling membrane [6], then it becomes evident that complete uncoupling in D-144 particles requires dissipation of both the H^+ gradient and the membrane potential. Furthermore, it is apparent from our data that in the previously proposed mechanism for the action of aliphatic amines in reversing uncoupling by valinomycin and NH_4Cl [10] the maintenance of the membrane potential in fact might play a key role, since the reversal effect is accompanied by a restoration of the light-induced signal at 518 nm.

Nelson et al. [24] showed that H^+ uptake was abolished in subchloroplast particles prepared with digitonin by a different procedure from that used here, whereas phosphorylation was retained. It would be of interest, therefore, to examine the light-induced absorbance change at 518 nm in these subchloroplast particles.

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References

[1] J.S.Neumann and A.T.Jagendorf, Arch. Biochem. Biophys. 107 (1964) 109.
[2] P.Mitchell and J.Moyle, Biochem. J. 105 (1967) 1147.
[3] L.-V.van Stedingk and H.Baltscheffsky, Arch. Biochem. Biophys. 117 (1966) 400.
[4] P.Mitchell, Nature 191 (1961) 144.
[5] P.Mitchell, in: Chemiosmotic Coupling and Energy Transduction, (Glynn Research, Bodmin, Cornwall, England, 1968).
[6] H.T.Witt, B.Rumberg and W.Junge, Colloq. Ges. Biol. Chem. 19 (1968) 262.
[7] D.E.Fleischmann and R.K.Clayton, Photochem. Photobiol. 8 (1968) 287.
[8] J.B.Jackson and A.R.Crofts, FEBS Letters 4 (1969) 185.
[9] R.E.McCarty, Biochem. Biophys. Res. Commun. 32 (1968) 37.
[10] R.E.McCarty, J. Biol. Chem. 244 (1969) 4292.
[11] A.R.Crofts, in: Regulatory Functions of Biological Membranes, ed. J.Jarnefeld (Elsevier, New York, 1968) p. 247.
[12] J.M.Anderson and N.K.Boardman, Biochim. Biophys. Acta 112 (1966) 403.
[13] R.E.McCarty and E.Racker, J. Biol. 242 (1967) 3435.
[14] G.A.Hauska, R.E.McCarty and E.Racker, Biochim. Biophys. Acta, in press.
[15] R.E.McCarty and E.Racker, Brookhaven Symp. Biol. 19 (1966) 202.
[16] J.R.Bolton, R.K.Clayton and D.W.Reed, Photochem. Photobiol. 9 (1969) 209.
[17] N.Shavit, R.A.Dilley and A.San Pietro, Biochemistry 7 (1968) 2356.
[18] D.C.Fork, J.Amesz and J.M.Anderson, Brookhaven Symp. Biol. 19 (1966) 81.
[19] M.Avron and B.Chance, Brookhaven Symp. Biol. 19 (1966) 149.
[20] R.S.Cockrell and E.Racker, Biochem. Biophys. Res. Commun. 35 (1969) 414.
[21] M.Montal, B.Chance and C.P.Lee, Biochem. Biophys. Res. Commun. 36 (1969) 428.

[22] A.Thore, D.L.Keister, N.Shavit and A.San Pietro, Biochemistry 7 (1968) 3499.
[23] J.B.Jackson, A.R.Crofts and L.-V.van Stedingk, European J. Biochem. 6 (1968) 41.
[24] N.Nelson, Z.Drechsler and J.Neumann, J. Biol. Chem. 245 (1970) 143.