TWO PROTONS TRANSFERRED PER ATP SYNTHESISED AFTER FLASH ACTIVATION OF CHROMATOPHORES FROM PHOTOSYNTHETIC BACTERIA

K. M. PETTY and J. B. JACKSON

Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, England

Received 6 November 1978

1. Introduction

The similarity between the ubiquinone/cytochrome *b/c* oxido-reductase of mitochondria and the analogous enzyme in chromatophores from photosynthetic bacteria has been discussed at length [1]. The ease with which electron equivalents may be delivered by pulsed light activation and counted has been exploited in the determination of accurate H^+/e ratios in chromatophores [2,3]. In this report we use the technique of single flash excitation to determine the stoichiometry of protons transported during ATP synthesis. It is pertinent to note that chromatophore ATPase is remarkably similar to its mitochondrial counterpart from the point of view of subunit composition [4], inhibitor sensitivity [5,6] and divalent cation requirement [7] so similar H^+/ATP stoichiometries may be expected.

We make use of the red shift of the carotenoid absorption spectrum of the chromatophores [8,9] and its decay to estimate how many charges are translocated during ATP synthesis. The ATP yield under similar conditions is determined by assay with luciferin/luciferase [10,11]. With chromatophores from 2 species and several strains of Rhodopseudomonad we find:

$$H^+/ATP = 2.25 \pm 0.16$$

2. Methods

Photosynthetic cells were grown and chromatophores were prepared by standard procedures [7]. *Rps. capsulata* was the organism of choice since it may be disrupted under milder conditions than *Rps. sphaeroides* (150 W sonication for 45 s in the presence of 0.2 mm diam. glass beads) to yield chromatophores with a routinely higher rate of photophosphorylation and more extensive effect of ADP on the carotenoid shift decay after a flash.

We have found that the phosphorylating activity of *Rps. sphaeroides* chromatophores may be improved if the bacterial cells are exposed to lysozyme (20 mg/10 g cell wt) in the presence of 1.3 mM EDTA at pH 8.0 for 30 min at 30°C (see [12]) before the sonication step (2 × 30 s) in the presence of 8 mM $MgCl_2$. Two such preparations were used in this report.

Carotenoid shift experiments and determination of reaction centre bacteriochlorophyll concentrations were performed on a dual wavelength spectrophotometer as in [13,14]. Photosynthesis was initiated with 20 μs half-peak width flashes spaced 50 s apart and events were averaged usually for 16 or 32 sweeps, for the single flash. Control experiments ensured that the dark time between flashes was sufficient to allow the electron transport reactions, the electrochemical proton gradient and the turnover of the ATPase to relax by >95%.

Luciferase was purified according to the method in [10] with firefly lanterns either purchased from Sigma, or collected by J. B. J. in Philadelphia. Luciferin from Calbiochem, was used without further purification. Light emission was measured on the same spectrophotometer as above with the monochromator slits completely closed. In wild-type chromatophores (St Louis and KB1) the photomultiplier was screened with a Schott OG550 cut-off

filter in addition to the usual saturated copper sulphate solution [13] to prevent interference from the carotenoid absorption band shifts. In the green mutants N22 and Ga this precaution was unnecessary. The emission signal was calibrated by the addition of a standard ATP solution within the linear range of the luciferin/luciferase.

The experiments were routinely performed in the presence of antimycin A to block electron flow through the ubiquinone/cytochrome b/c_2 oxidoreductase and abolish carotenoid shift phase III [15]. This facilitates measurement of the extent of the carotenoid shift decay stimulated by ADP but, in single flash experiments, only slightly inhibits (between 20–40%) the ATP yield [8,9] (K. M. P., J. B. J. unpublished observations).

We are grateful to Dr J. H. Klemme for a culture of the KB1 strain and to Dr O. T. G. Jones for the N22 mutant.

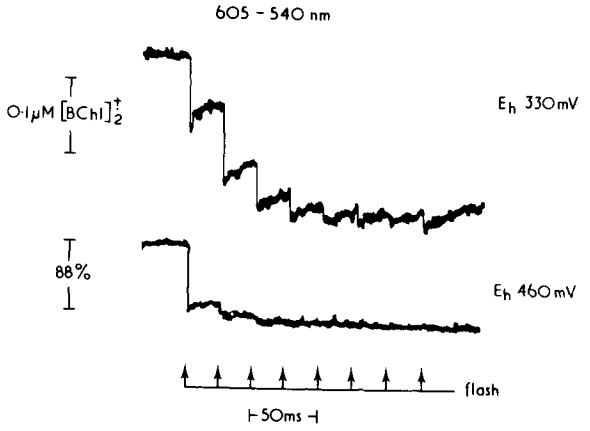

Fig.1. $[BChl]_2$ oxidation following a train of 8 saturating xenon flashes at 25 ms intervals monitored at 605–540 nm. The *Rps. sphaeroides* Ga chromatophores ($[BChl]_2$:0.2 μM) were suspended in 100 mM glycylglycine, 100 μM succinate, 0.1% BSA, 10 mM Mg^{2+}, 2 mM P_i, 5 μM antimycin at pH 7.75. Ferri/ferrocyanide (~5 μM) was used to adjust the ambient redox potential to the values shown. The traces represent the average of 8 scans spaced 50 s apart.

3. Results

3.1. *Method for the determination of H^+/ATP following a short flash*

Following single turnover flash activation of chromatophores in the presence of antimycin A electrons are transferred from reaction centre bacteriochlorophyll, through an intermediate, I, to a ubiquinone species, Q.Fe [16]. Subsequent electron transfer to a secondary ubiquinone results in proton binding (1 H^+/e^-) on the outside interface of the chromatophore vesicles [2]. The oxidised reaction centre bacteriochlorophyll is re-reduced by electron transport from cytochrome c_2 which is located on the inside of the chromatophore lumen [17]. These rapid electron transfer processes (complete within 1 ms) are seen to take place across the membrane. The number of charges translocated may be estimated from the $\Delta A_{606-540\ nm}$ due to reaction centre bacteriochlorophyll [14]. Rapid re-reduction of reaction centre bacteriochlorophyll by cytochrome c_2 would lead to an underestimate of this parameter but repetitive activation by closely spaced flashes in the presence of antimycin A to block cyclic electron flow circumvents this problem. Allowance for incomplete flash saturation is made by comparison with data at high ambient redox potential where cytochrome c_2 is chemically oxidised before the flash (see fig.1). The table shows the number of electrons transferred by a single flash (on a bulk bacteriochlorophyll basis) in several chromatophore preparations. These values fall within the same range as those measured in *Rps. sphaeroides* chromatophores [14].

The separation of charge across the chromatophore membrane results in a red shift of the endogenous carotenoid absorption spectrum. The shift is linear with respect to the applied voltage so at constant membrane capacitance, the extent of the shift, measured as an absorbance change at the peak and trough of the difference spectrum, is proportional to the number of charges transferred (20) as calculated above.

A fraction of the decay of the carotenoid shift is accelerated under phosphorylating conditions [8,9] (fig.2). Evidence suggests that this is a consequence of charge flux through the chromatophore ATPase during the phosphorylation of added ADP. The extent of charge flux through the ATPase, expressed as a percentage of the total number of charges translocated through the reaction centre may be estimated from semilogarithmic plots of the decay of the carotenoid

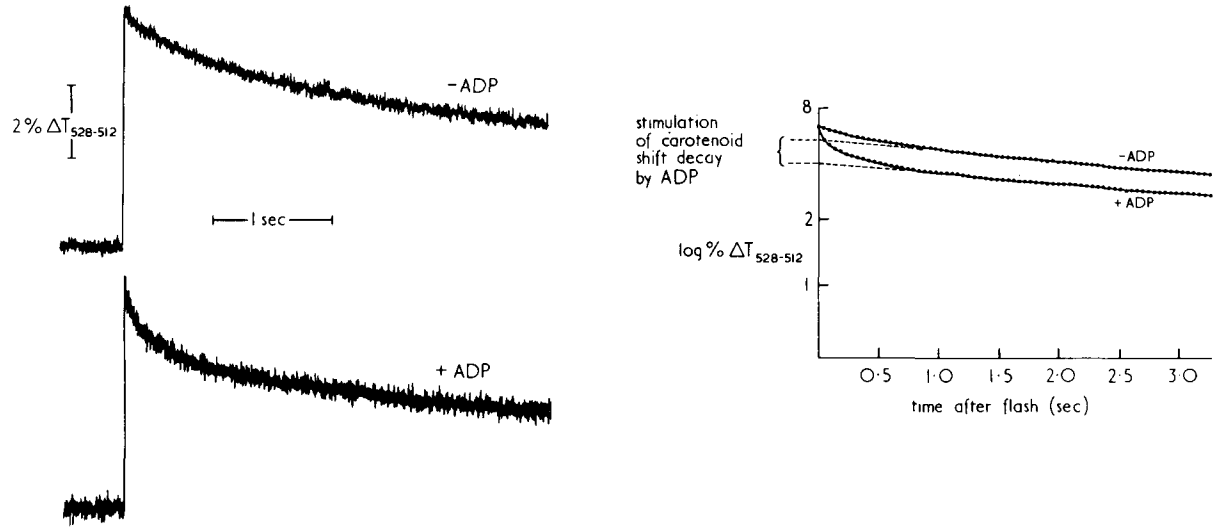

Fig.2. The carotenoid bandshift and decay: Chromatophores from *Rps. capsulata* KB1 were suspended in the medium described in the legend to fig.1. In the lower left hand trace 30 μM ADP was present. The measuring wavelengths were 528–512 nm. The traces were averaged as in fig.1. On the right hand side is shown a semi-logarithmic plot of the data on the left. The addition of ADP has resulted in a 22% stimulation of the decay.

Table 1
Determination of H^+/ATP ratios in several preparations of *Rhodopseudomonas capsulata* and *sphaeroides*

Chromatophores prepared from	Conditions	$[BChl]^+_2$/BChl on single turnover	% Acceleration of carotenoid shift decay by ADP	Charges moving through ATPase/BChl	ATP synthesis/ flash/BChl	$\frac{H^+}{ATP}$
Rps. capsulata strain KB1	200 μM ADP 5 μM antimycin	1/54	17	1/323	1/698	2.16
Rps. capsulata strain KB1	200 μM ADP 5 μM antimycin 120 ng/ml efrapeptin	1/54	5	1/1080	1/2333	2.16
Rps. capsulata St Louis	20 μM ADP	1/78	15	1/520	1/1071	2.06
Rps. capsulata N22	150 μM ADP 5 μM antimycin	1/125	19	1/658	1/1665	2.53
Rps. capsulata N22	30 μM ADP 5 μM antimycin	1/98	13	1/754	1/1734	2.30
Rps. capsulata N22	30 μM ADP 5 μM antimycin	1/200	18	1/1111	1/2477	2.23
Rps. capsulata N22	30 μM ADP	1/125	27	1/458	1/1058	2.31
Rps. capsulata N22	30 μM ADP	1/125	19	1/658	1/1316	2.00
Rps. sphaeroides Ga	30 μM ADP 5 μM antimycin	1/106	11	1/964	1/2155	2.24
Rps. sphaeroides Ga	30 μM ADP	1/82	8	1/1026	1/2564	2.50
					mean =	2.25 ±0.16

shift (fig.2) and is shown in table 1. In the adjacent column these values are converted into the absolute number of charges passing through the ATPase on a bulk bacteriochlorophyll basis using the figures in the first column.

Immediately after each carotenoid experiment, the monochromator slits of the spectrometer were closed and a fresh chromatophore sample in the same medium, supplemented with luciferin and purified luciferase, was analysed for flash-induced ATP synthesis. Light emission in the absence of added ADP (about 10% of that in the presence of 30–200 μM added ADP), presumably due to endogenous nucleotide, was subtracted in the computation of the flash yields of ATP synthesis given in table 1 (see also fig.3). The flash-induced phosphorylation of 'endogenous' nucleotide probably partly accounts for the initial fast decay phase of the carotenoid shift in the absence of ADP (see fig.2).

The final column shows the H^+/ATP ratio for each preparation. The mean value is 2.25 ± 0.16 H^+/ATP. In a chromatophore sample, 60% inhibited with efrapeptin, a similar value was obtained. In three experiments antimycin was omitted from the chromatophore suspension. In such cases the stimulation of the carotenoid shift was difficult to determine accurately from the log plots but an approximate value was estimated as the difference (± ADP) in the carotenoid shift level 500 ms after the flash.

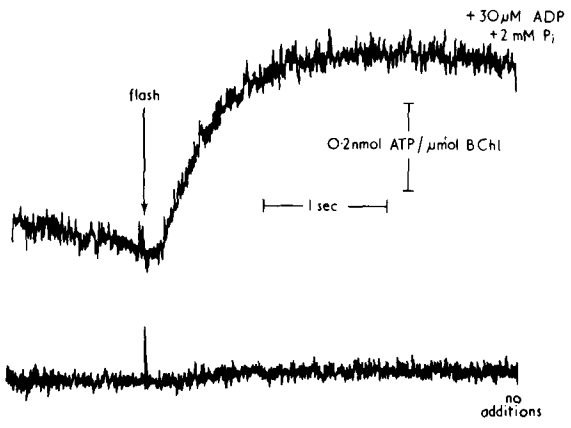

Fig.3. ATP synthesis following a single turnover flash in the presence of 5 μM antimycin. The medium of fig.1 was supplemented with 0.2 mg purified luciferase, 150 μM luciferin, and in the upper trace, 30 μM ADP. Light emission (upwards) was calibrated by addition of 10 nM ATP.

3.2. *Possible sources of error in the determination of H^+/ATP*

(i) Although these experiments were largely conducted with chromatophores prepared from *Rps. capsulata* (for the reason given in section 2) the extinction coefficient for the reaction centre change at 605–540 nm was derived for *Rps. sphaeroides* chromatophores [14]. In view of the similarities between the two organisms, we doubt that the error introduced by this assumption is significant.

(ii) The kinetics and redox potential dependence of the generation and decay of the flash-induced carotenoid shift are entirely consistent with the thesis that the change is a response to charge translocation across the chromatophore membrane [8,9,13,20,21]. Detailed spectral analysis are generally supportive of an electrochromic mechanism [22,23]. Discrepancy does arise between membrane potential determinations by either carotenoid shift measurements or by ion uptake techniques [24–27], the former giving larger values. This comparison however suffers from the fact that, to date, the ion-uptake experiments have apparently been carried out at less than saturating light intensities. In any case, in the present experiments, we do not rely on diffusion potential calibrations of the carotenoid shift but rather upon the equivalence between the extent of the band shift and the quantity of charge translocated by either electron transport or through the ATPase.

(iii) On average only 60% of the reaction centres in our chromatophore preparations possess functionally intact cytochrome c_2 [14,21]. The remainder appears to be lost during cell disruption. This means that, in 40% of the reaction centres, electronic charge crosses only a part of the membrane [20,21,28]. Consequently we would be over-estimating the charge translocated through both the electron transport reactions and the ATPase complex by about 25%, giving an average H^+/ATP ratio = 1.7. In fact the error introduced by this problem is $< 25\%$ owing to the remarkable observation that at high redox potentials, where all the cytochrome c_2 is chemically oxidised before the flash and electron transport only occurs across a part of the mem-

brane (viz. $[BChl]_2 \rightarrow Q.Fe.$), ATP synthesis still proceeds after the flash [21]. The implications of this finding will be discussed in a future communication so suffice it to say that considerations do not seriously affect the computed ratio.

4. Discussion

The yield of ATP synthesis after a single turnover, nearly saturating flash is usually of the order of 1/700 molecules of bacteriochlorophyll. In [29] 1/1000 was obtained in chromatophores of *Rhodospirillum rubrum.* Our chromatophores from *Rps. capsulata* possess a mean 1000 molecules of bulk bacteriochlorophyll and 11 electron transport chains/vesicle [19]. Thus ATP synthesis, at least after single flash activation, would appear to be rather inefficient, a fact which was also deduced from studies on the carotenoid shift decay under phosphorylating conditions [8,9]. Increased ATP synthesis on subsequent flashes has been reported in chloroplasts [30] and has also been discovered in chromatophores ([29], K. M. P., J. B. J. unpublished observations). In the present experiments we arranged the dark time between flashes to be so long that the apparent 'activation' processes completely decayed so that signal averaging was possible.

Before the advent of purified luciferase preparation the error in single flash yields of ATP synthesis determined by incorporation of $^{32}P_i$ was too large to be of value for measurement of the H^+/ATP ratio. A less direct method gave a value of $H^+/ATP = 1.7$ with a rather large standard deviation [9]. The present work suggests with more confidence that the ratio is 2.

In the absence of antimycin A, 2 H^+ are bound to the outer interface of the chromatophore membrane per e^- delivered to the ubiquinone/cytochrome b/c_2 oxidoreductase [3] and in the presence of antimycin, 1 H^+/e^- [2]. Although, for as yet unknown reasons, not all electron transfer reactions couple to ATP synthesis after single flash activation, the effective coupling stoichiometry for phosphorylation in the absence of antimycin is 1 e^-/2 H^+/1 ATP.

Acknowledgements

We are grateful to Nick Cotton and Julia Greenrod for maintenance of the bacterial cultures and the preparation of chromatophores. Financial support was received from the SRC.

References

[1] Dutton, P. L. and Wilson, D. F. (1974) Biochim. Biophys. Acta 346, 165–212.
[2] Petty, K. M. and Dutton, P. L. (1976) Arch. Biochem. Biophys. 172, 335–345.
[3] Petty, K. M., Jackson, J. B. and Dutton, P. L. (1977) FEBS Lett. 84, 299–303.
[4] Johansson, B. C. and Baltscheffsky, M. (1975) FEBS Lett 53, 221–224.
[5] Baccarini-Melandri, A., Fabbri, E. and Melandri, B. A. (1975) Biochim. Biophys. Acta 376, 82–88.
[6] Webster, G. D. and Jackson, J. B. (1978) Biochim. Biophys. Acta 503, 135–154.
[7] Edwards, P. A. and Jackson, J. B. (1976) Eur. J. Biochem. 62, 7–14.
[8] Saphon, S., Jackson, J. B. and Witt, H. T. (1975) Biochim. Biophys. Acta 408, 67–82.
[9] Jackson, J. B., Saphon, S. and Witt, H. T. (1975) Biochim. Biophys. Acta 408, 83–92.
[10] Nielsen, R. and Rasmussen, H. (1968) Acta Chem. Scand. 22, 1757–1762.
[11] Lundin, A., Thore, A. and Baltscheffsky, M. (1977) FEBS Lett. 79, 73–76.
[12] Miura, T. and Mizushima, S. (1969) Biochim. Biophys. Acta 193, 268–276.
[13] Jackson, J. B. and Crofts, A. R. (1971) Eur. J. Biochem. 18, 120–130.
[14] Dutton, P. L., Petty, K. M., Bonner, H. S. and Morse, S. D. (1975) Biochim. Biophys. Acta 387, 536–556.
[15] Prince, R. C. and Dutton, P. L. (1977) Biochim. Biophys. Acta 462, 731–747.
[16] Dutton, P. L., Prince, R. C., Tiede, D. M., Petty, K. M., Kaufmann, K. J., Netzel, T. L. and Rentzepis, P. M. (1976) Brookhaven Symp. Biol. 28, 213–237.
[17] Prince, R. C., Baccarini-Melandri, A., Hauska, G. A., Melandri, B. A. and Crofts, A. R. (1975) Biochim. Biophys. Acta 387, 212–227.
[18] Jackson, J. B. and Crofts, A. R. (1969) FEBS Lett. 4, 185–189.
[19] Packham, N. K., Berriman, J. A. and Jackson, J. B. (1978) FEBS Lett. 89, 205–210.
[20] Jackson, J. B. and Dutton, P. L. (1973) Biochim. Biophys. Acta 325, 102–113.
[21] Jackson, J. B., Greenrod, J. A., Packham, N. K. and Petty, K. M. (1978) in: Frontiers of Biological Energetics, Academic Press, New York, in press.
[22] De Grooth, B. G. and Amesz, J. (1977) Biochim. Biophys. Acta 462, 237–246.
[23] Symons, M., Swysen, C. and Sybesma, C. (1977) Biochim. Biophys. Acta 462, 706–718.

[24] Kell, D. B., Ferguson, S. J. and John, P. (1978) Biochim. Biophys. Acta 502, 111–126.
[25] Kell, D. B., John, P. and Ferguson, S. J. (1978) Biochem. J. 174, 257–266.
[26] Michels, P. A. M. and Konings, N. N. (1978) Eur. J. Biochem. 85, 147–155.
[27] Michels, P. A. M. and Konings, W. N. (1978) Biochim. Biophys. Acta 507, 353–368.
[28] Takamiya, K. and Dutton, P. L. (1977) FEBS Lett. 80, 279–284.
[29] Baltscheffsky, M. (1978) abst. 86, p. 17, Proc. Biophys. Cong. Japan.
[30] Harris, D. A. and Crofts, A. R. (1978) Biochim. Biophys. Acta 502, 87–102.