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CORRELATION BETWEEN ATP SYNTHESIS AND THE DECAY OF THE CAROTENOID BAND SHIFT AFTER SINGLE FLASH ACTIVATION OF CHROMATOPHORES FROM *RHODOPSEUDOMONAS CAPSULATA*

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Summary

ATP synthesis and the acceleration of the decay of the carotenoid absorption band shift after single flash excitation of *Rhodopseudomonas capsulata* chromatophores were compared. The two processes behave similarly with respect to: (1) ADP and P_i concentration; (2) inhibition by efrapeptin and venturicidin, and (3) inhibition by valinomycin/K^+ and by ionophores.

Taken together with earlier evidence for the electrochromic nature of the carotenoid band shift the data support the contention that positive charge moves outwards across the chromatophore membrane during ATP synthesis and justify the method for determination of the H^+/ATP ratio (Petty, K.M. and Jackson, J.B. (1979) FEBS Lett. 97, 367—372).

The ability of nucleotide diphosphates in the presence of P_i and Mg^{2+} to give rise to the acceleration of the carotenoid shift decay closely correlates with the rate of phosphorylation of the nucleotides in steady-state light. Nucleotide triphosphates enhance the decay in parallel with their rate of hydrolysis.

Adenylyl imidodiphosphate is itself without effect on the decay of the carotenoid shift and it does not prevent the ADP-induced acceleration. The analogue does prevent the ATP effect but only after repeated flashes.

Introduction

The use of pulsed light activation in the study of electron transport, H^+ binding and membrane potential development in photosynthetic organelles [1] complements the steady-state type of measurements usually encountered in work on oxidative electron transport in mitochondria. Until recently an imme-

diate and in situ assay of ATP concentration was unavailable so that studies on ATP synthesis following single turnover flashes were indirect [2]. The development of a simple purification procedure for the enzyme luciferase represents a considerable advance in the determination of low concentrations of ATP since light emission with enzyme and purified luciferin remains constant over long periods [3,4]. The assay components may be included in the organelle suspension without deleterious effects on the ATP-synthesising machinery [4].

The mechanism by which the electrochemical potential of protons generated during electron transport is coupled to ATP synthesis is not understood. In chromatophores from photosynthetic bacteria the development and decay of an apparently transmembrane electric potential may be followed from the shift in absorption of endogenous carotenoid pigments [5—10]. Under conditions appropriate for ATP synthesis the decay of the shift is partially accelerated and this has been attributed to the outward flux of H^+ through the membrane-bound ATPase [11]. The initial aim of the experiments described in this paper was to investigate whether the accelerated decay of the carotenoid shift with ADP was modified in concert with ATP synthesis measured with luciferin/luciferase. The correlation proved to be sound. Since the accelerated carotenoid shift decay is an immediate indicator of energy utilisation during ATP synthesis, we are able to make some deductions as to the steps of energy input during the overall catalysis by the ATPase after single flash excitation.

Methods

Photosynthetic cells of *Rhodopseudomonas capsulata* were grown and chromatophores were prepared by sonication by the usual methods [2]. The rate of ATP synthesis in continuous illumination with 1 mM succinate at pH 7.4 was routinely 1—3 mol/mol bacteriochlorophyll per min. Luciferase was prepared from firefly lanterns according to published procedures [3,4] but luciferin was purchased from Calbiochem.

Shifts in the carotenoid absorption spectrum were measured at fixed wavelengths (see figures legends) with either a single-beam or double-beam spectrophotometer as previously descrbed [2,6]. The exciting flash had a half-peak width of 20 μs and was more than 85% saturating at bacteriochlorophyll concentrations of 20 μM. The carotenoid shift kinetics were usually averaged over 8, 16 or 32 flashes. We found that it was necessary to keep the dark time between flashes to a minimum of about 50 s to prevent accumulation of photoproducts.

Light emission from the luciferase/luciferin/ATP reaction after flash excitation of the chromatophores was measured in the same spectrophotometer but with the monochromator slits closed and, in the case of wild-type strains, with an additional cut-off filter, Schott OG-550 shielding the photomultiplier. Averaging was usually unnecessary for the light emission signal but was acceptable provided the flash frequency was less than 0.02 Hz. The light emission was calibrated by the addition of standard ATP solution.

Antimycin A, valinomycin, carbonylcyanide trifluoromethyoxyphenyl hydrazone and nucleotides were purchased from Sigma, London. Efrapeptin was a gift from Clive Jackson, Shell Research, Sittinghbourne.

Results

The decay of the carotenoid absorption band shift after short flash excitation of *Rps. capsulata* (strain KB1) chromatophores has contributions from three processes [10]. Slowest, with a half-time = 10 s is a component which is most probably a response to the electrophoretic transport of ions down the membrane potential gradient generated by the flash. A component ($t_{1/2}$ = 1 s) corresponds to a back transfer of electrons into reaction centre chlorophyll. In the presence of phosphorylation substrates a very rapid decay component ($t_{1/2}$ = 5—10 ms) increases in magnitude from 1 to 2% of the total to around 20% depending on the preparation. We have argued that, since high concentrations of uncoupling agents or ionophores accelerate the entire decay of the carotenoid shift into a purely monotonic curve, all three of the decay processes act on a single intermediate, namely the chromatophore membrane potential [10]. On this basis, the three components, may be resolved from semi-logarithmic plots of the carotenoid decay as though they contribute separately to the decay of the membrane potential.

The fastest of the three decay phases, the one which we are primarily concerned with in this report, increases in extent with increasing ADP concention while its half-time remains constant (Figs. 1 and 2). The ADP concentration for half-maximal effect in strains N22 and St. Louis of *Rps. capsulata* is approximately 3 μM, in close agreement with the earlier report in *Rps. sphaeroides* [11]. In chromatophores prepared from the KB1 strain of *Rps. capsulata*, which has a greater rate of steady-state light-induced ATP synthesis than the N22 and St. Louis strain [12], the stimulation of the carotenoid shift decay by ADP is no more extensive although a routinely higher concentration of ADP (25 μM) was necessary for half-maximal effect.

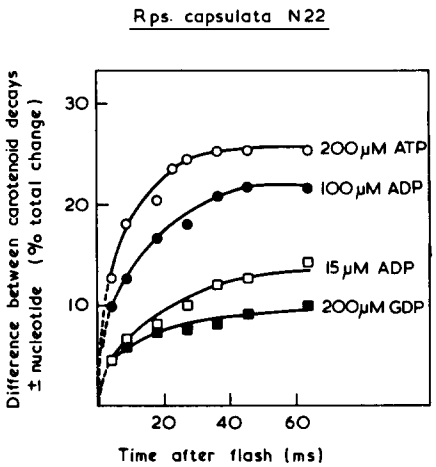

Fig. 1. Kinetics of the nucleotide phosphate-induced acceleration of the carotenoid shift decay after single flash activation. Chromatophores from *Rps. capsulata*, strain N22, were suspended at a concentration of 22 μM bacteriochlorophyll in a medium containing 10% sucrose, 50 mM KCl, 50 mM Tricine, 5 mM $MgCl_2$, 2 mM K_2HPO_4, 5 μM antimycin, final pH 8.0, in the absence or presence of nucleotide at the indicated concentration. The averaged decay of the carotenoid shift (503—587 nm) was recorded after 16 flashes, 50 s apart. The traces (presence and absence of nucleotide) were superimposed and the difference was calculated manually and expressed as a percentage of the total carotenoid change.

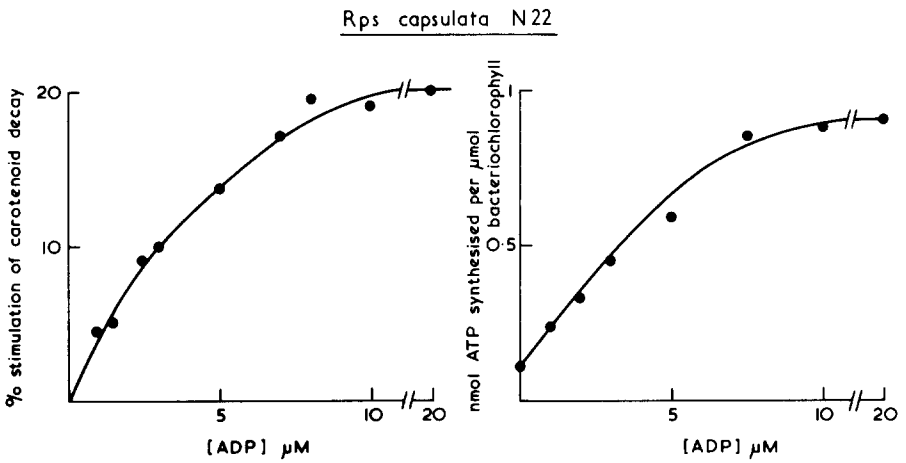

Fig. 2. Similar ADP requirement for acceleration of carotenoid shift decay and ATP synthesis after single flash activation. Left. Chromatophores from *Rps. capsulata*, strain N22, were suspended at a concentration of 20 μM bacteriochlorophyll in 2.5 ml of a medium of 100 mM glycylglycine, 50 mM KCl, 10 mM magnesium acetate, 100 μM sodium succinate, 0.1% bovine serum albumin, 2 mM phosphate, 5 μM antimycin, final pH 7.75, in the presence of ADP at the concentration indicated. The averaged decay of the carotenoid shift (503—587 nm) was recorded after 16 flashes, 50 s apart. The percent stimulation of the carotenoid shift decay is defined and estimated from semi-logarithmic plots as in Ref. 20. Right. Chromatophores at the same concentration were suspended in a similar medium supplemented with 150 μM luciferin and 0.2 mg purified luciferase. ATP synthesis after flash activation was recorded as described in Methods.

Under similar conditions to those described above but in the presence of 0.15 mM luciferin and 0.2 mg luciferase, a burst of ATP synthesis may be observed from the light emission following the flash (Fig. 2). The amount of ADP required for a half-maximal flash yield of ATP closely coincides with that required for carotenoid shift acceleration. The rate of light emission following the flash ($t_{1/2}$ = 0.15 s) is slower than the ADP-induced carotenoid response but is almost certainly limited by the rate of reaction between ATP and luciferase [4,13]. It is, however, quite clear that the period of light emission accompanying ATP synthesis is complete before the electrochemical proton gradient (indicated by the carotenoid shift and pH indicator record, see Ref. 2) have half-decayed.

The F_1-ATPase inhibitor efrapeptin decreases, in parallel, the ATP flash yield, the acceleration of the carotenoid shift by ADP (Fig. 3) and steady-state light-induced phosphorylation. The apparent rate constants for the luciferin/luciferase-dependent light emission and for the ADP/P_i-dependent component in the decay of the carotenoid shift are however, not affected by efrapeptin. Efrapeptin is completely without effect on the ADP-independent processes in the carotenoid shift decay. Although our efrapeptin sample is a mixture (Jackson, C., personal communication), if we assume a molecular weight of 1650 the titration yields a value of one molecule of efrapeptin/250 bacteriochlorophyll for 50% inhibition, an approximate upper limit for the amount of ATPase in the chromatophore preparation. The F_1F_0-ATPase inhibitor, venturicidin also has a potent and parallel inhibitory effect on the ATP yield and the ADP-accelerated carotenoid shift. In *Rps. capsulata* chromatophores

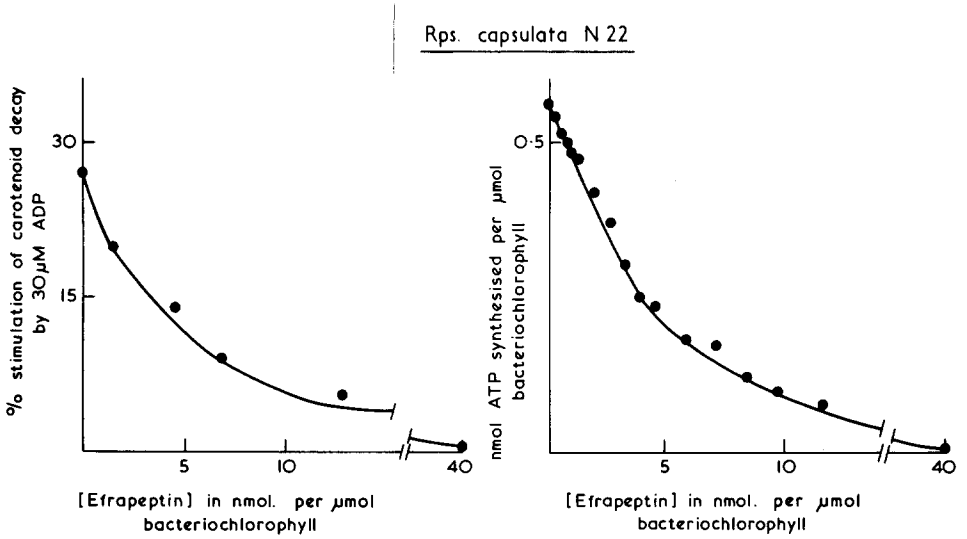

Fig. 3. Parallel inhibition of ADP-accelerated carotenoid shift decay and ATP synthesis by efrapeptin. The experiments were performed as described in Fig. 2. Efrapeptin was added in 1—20-μl aliquots of ethanolic solution. The ethanol alone had no effect.

venturicidin and efrapeptin are the most potent of the ATPase inhibitors that we have tested (oligomycin, citreoviridin and aurovertin). None of these energy transfer inhibitors affect the amplitude of the carotenoid shift following flash activation of antimycin-treated chromatophores (see also [11]).

The ionophorous antibiotic, valinomycin (Fig. 4) and the uncoupling agent, carbonylcyanide trifluoromethoxyphenyl hydrazone (data not shown) also have a parallel inhibitory effect on the single flash yield of ATP and on the accelerated decay of the carotenoid shift by ADP. Valinomycin inhibition is strictly K^+ dependent. The concentrations required for 90% inhibition are

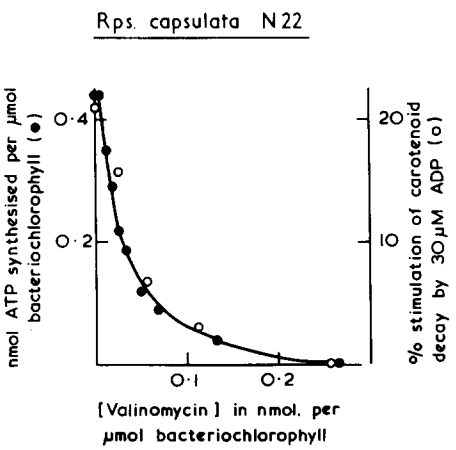

Fig. 4. Parallel inhibition of ADP-accelerated carotenoid shift decay and ATP synthesis by valinomycin. The experiments were performed as described in Fig. 2.

exceedingly low, about one molecule of valinomycin/5000 molecules of bacteriochlorophyll. At this concentration of ionophore, in the absence of ADP, the slow decay phase of the carotenoid shift, thought to correspond to the electrophoretic transfer of ions across the chromatophore membrane is accelerated [2,10].

Nucleotide specificity for the acceleration of the carotenoid shift decay after a single flash

The extent by which various nucleotide diphosphates (all at 200 μM) are able to stimulate the decay of the carotenoid shift after a single flash is shown in Table I. The concentration of GDP and IDP required for half-maximal effect is greater than that for ADP but the kinetics of the acceleration are essentially similar with all three nucleotides. The specificity of the luciferin/luciferase reaction prevents us from directly comparing the chromatophore specificity for flash-induced nucleotide diphosphate phosphorylation with the acceleration of the carotenoid shift decay. The nucleotide specificity of the phosphorylation reaction during continuous illumination can be investigated from the rate of H^+ disappearance measured with a glass electrode. Table I shows that except for the inverted order of GDP and IDP the nucleotide requirement of phosphorylation is similar to that for the accelerated decay of the carotenoid shift. The specificity for nucleotides is also quite similar to that described for mitochondrial particles [14]. Moreover the concentrations required for half-maximal stimulation of the carotenoid shift compare favourably with the K_m values for mitochondrial phosphorylation [15].

TABLE I

NUCLEOTIDE SPECIFICITY OF THE ACCELERATED CAROTENOID SHIFT DECAY AND NUCLEOTIDE TRIPHOSPHATE SYNTHESIS AND HYDROLYSIS IN *RPS. CAPSULATA*, STRAIN ST. LOUIS

For percent stimulation of carotenoid shift decay see Fig. 2. Rate of phosphorylation was measured from rate of H^+ disappearance in unbuffered chromatophore suspension [17]. Rate of hydrolysis was measured by colorimetric assay of phosphate release [17].

Nucleotide	% stimulation of carotenoid shift decay by 200 μM nucleotide	Concentration of nucleotide for half-maximal effect	Rate of phosphorylation of 100 μM nucleotide in continuous light (μmol ATP/μmol bacteriochlorophyll per min)	Rate of hydrolysis of 1 mM nucleotide (μmol ATP/μmol bacteriochlorophyll min)
ADP	18	3	2.55	—
IDP	4	—	2.25	—
GDP	9	80	1.99	—
UDP	0	—	1.02	—
CDP	0	—	0.11	—
ATP	22	8	—	1.06
ITP	11.5	125	—	0.29
GTP	12	5	—	0.23
UTP	10 *	360	—	0.14
CTP	5.5 *	220	—	0.11

* Using 500 μM nucleotide.

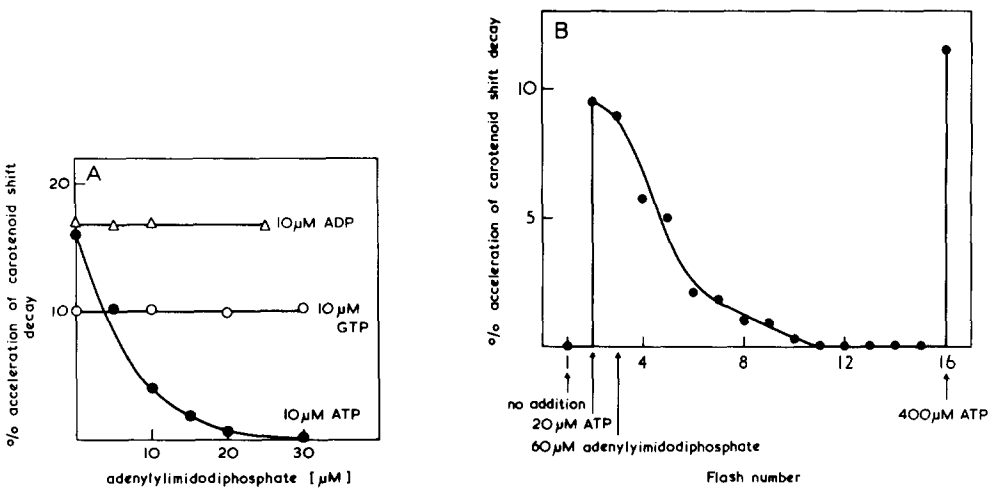

Fig. 5. The effect of adenylyl β,γ-imidodiphosphate on the acceleration of the carotenoid shift decay by nucleotide phosphate. (A) Conditions as Fig. 2. The adenylyl imidodiphosphate was added at the same time as the substrate nucleotide phosphate, about 1 min before flash excitation was started. The acceleration of the carotenoid shift decay by nucleotide phosphate was obtained from averaged traces after 16 flashes. (B) Experimental suspension medium as Fig. 2. The carotenoid shift decay after the first flash was recorded in the absence of nucleotide and defined as zero acceleration. About 5 min after the first flash, 20 μM ATP was added and the carotenoid shift after the second flash was recorded. About 5 min after the second flash, 60 μM adenylyl imidodiphosphate was added and the carotenoid shift decay after the third flash was recorded. Flashes were then repeated at 5-min intervals and the carotenoid shift was appropriately recorded. After the fifteenth flash, 400 μM ATP was added and the final flash was fired. For each carotenoid shift recording the accelerated decay was estimated with respect to the first flash from semilogarithmic plots.

The nucleotide triphosphates are also capable of giving rise to an acceleration of the carotenoid shift decay after a single flash and with similar kinetics to the diphosphates. The extent of the stimulation is usually greater with the triphosphate than with the diphosphate. The specificity for different nucleotides, however, is similar (Table I).

As with mitochondrial particles [16], the nucleotide analogue adenylyl imidodiphosphate inhibits chromatophores ATP hydrolysis at much lower concentrations than it affects ATP synthesis (Greenrod, J.A. and Jackson, J.B., unpublished observations). Furthermore, the analogue up to concentrations of 0.2 mM was without effect on the single flash yield of ATP synthesis measured with luciferase. Fig. 5A shows that adenylyl imidodiphosphate at low concentrations prevents ATP but not ADP or GTP from giving rise to the accelerated decay of the carotenoid shift. The analogue itself, in the absence of added nucleotides did not modify the decay kinetics of the carotenoid shift. These experiments were carried out under repetitive flash conditions (0.02 Hz) with signal averaging. After a single flash the nucleotide-induced acceleration of the carotenoid shift decay is measurable despite the poor signal/noise ratio (Fig. 5B). An examination of subsequent flashes at 5-min intervals showed that the stimulation by ATP was independent of the number of flashes given. In the experiments shown in Fig. 5B, adenylyl imidodiphosphate scarcely blocked the acceleration of the carotenoid shift induced by ATP on the first flash following

the addition of the analogue (9% acceleration compared with 9.6% in the absence of adenylyl imidodiphosphate). Inhibition became marked after later flashes. The competitive nature of the inhibitor is revealed on the 16th flash where excess ATP again gives rise to a stimulated decay.

The nucleotide-induced acceleration of the carotenoid shift decay was in all cases strictly dependent on the presence of Mg^{2+} in the suspension medium. Addition of 0.5 mM EDTA completely suppressed the effect but restoration was achieved by titrating against $MgCl_2$. The effects of Mg^{2+} are complex, however, and have not yet been examined in great detail. We have found that the concentration of nucleotide required for half-maximal stimulation of the carotenoid shift decay is dependent on the $MgCl_2$ concentration in a manner which is not simply indicative of a Mg^{2+}-nucleotide substrate complex. For instance a half-maximal effect is achieved with 30 μM ATP in a medium containing 0.5 mM $MgCl_2$ and with 8 μM ATP in the presence of 8 mM $MgCl_2$. At both $MgCl_2$ concentrations but in the presence of the 'activating anion' sulphite (2 mM), ATP is half-effective at 8 μM. Such data are reminiscent of the opposing effects of Mg^{2+} and SO_3^{2-} in controlling steady-state rates of ATP hydrolysis in *Rhodospirillum rubrum* chromatophores [17]. It may be noted that in the wide range of nucleotide, Mg^{2+} and sulphite concentrations that we have employed the extent of stimulation of the decay under optimal conditions is never more than 20—30% of the total carotenoid shift produced by the flash.

The stimulation of the carotenoid shift decay by nucleotide di- and triphosphates (ADP, ATP and GTP were examined in detail) is also phosphate dependent but phosphate has no effect in the absence of nucleotide. The phosphate concentration dependence of the carotenoid effect and of the single flash yield of ATP synthesis are similar. Both are half-maximal at about 0.5 mM phosphate. Arsenate at approximately five-fold higher concentrations may substitute for phosphate in accelerating the carotenoid shift decay but sulphite up to 20 mM is ineffective.

Discussion

The coincidence of the flash-induced ATP synthesis measured with luciferin/luciferase and the accelerated decay of the carotenoid absorption band shift is supported by (i) similar dependence on ADP concentration; (ii) similar dependence on P_i concentration; (iii) parallel inhibition by an F_1 inhibitor, efrapeptin and an F_0 inhibitor, venturicidin, and (iv) parallel inhibition by ionophores. In addition, the nucleotide specificity of the carotenoid shift effect is similar to that of steady-state light-induced phosphorylation and dark hydrolysis.

There are currently two interpretations of the carotenoid absorption band shifts in chromatophores. Both points of view accept the electrical origin of the response [8,9] but opinions differ as to which fields, generated by electron transport and associated protolytic reactions, are effective. On the one hand, the pigments may respond to delocalised potentials across the chromatophore membranes [6,10,18], or, alternatively, there may be contributions from local electric fields arising from charge separations between adjacent electron transport carriers (Ref. 7, and Symons, M. and Crofts, A.R., personal communication). Based on the coincident spectra of the different phases of the generation

and decay of the absorption shift and on the fact that ionophores and uncoupling agents accelerate the decay of the entire process, we subscribe to the former, simpler interpretation [10]. The data presented above would therefore support the contention that the accelerated decay of the band shift after a flash in the presence of phosphorylation substrates is a consequence of electrophoretic displacement of charge accompanying ATP synthesis (see [19]), and justifies some of the assumptions made in our quantitative estimates of the number of charges translocated/ATP synthesised after single flash excitation [20].

However, based on their observation that 1-N^6-ethenoadenosine diphosphate (ϵ-ADP), which is phosphorylated during continuous illumination, does not give rise to an accelerated decay of the analogous 515 nm shift after single flash excitation of chloroplast suspensions, Girault and Galmiche [21] concluded that this hypothesis is untenable. We have repeated the experiments with ϵ-ADP in chromatophores and obtained similar results (unpublished results). Girault and Galmiche suggested that, since ATP is effective in promoting the decay of the 515 nm shift, the phenomenon is due to an interaction between ATP and the ATPase which, in an unspecified way, modifies the carotenoid response. The strict phosphate requirement in chromatophores, makes this unlikely and it may be worth exploring other explanations of the data obtained with ϵ-ADP. Unfortunately, the nucleotide specificity of the luciferase reaction prohibits us from measuring single flash yields of ϵ-ATP synthesis and we can only speculate on likely possibilities. It is significant in this connection that the binding affinities of ADP and ϵ-ADP to chloroplast CF_1 depend differently on the ambient Mg^{2+} concentration [22]. This parameter is known to modify the state of activity of ATPase [17]. The state of the ATPase during single flash activation may be different to that during steady-state illumination [27]. With these points in mind we think it not unlikely that ϵ-ATP synthesis does not occur or proceeds very slowly under conditions of flash illumination. ϵ-ATP is not appreciably hydrolysed under steady-state conditions and does not give rise to an accelerated carotenoid decay.

With this reservation we shall return to the proposed model and discuss the results accordingly. The data obtained with the nucleotide triphosphates make a useful point of departure. ATP hydrolysis by darkened chromatophores is slow [23] and is probably limited by release of ADP from the ATPase [24]. So that even in the presence of added ATP, the enzyme is predominantly loaded with the nucleotide diphosphate. The requirement for added P_i for ATP-dependent acceleration of the carotenoid shift decay after a flash further suggests that during dark ATP hydrolysis, P_i release precedes rate-limiting ADP release. We can rule out indirect effects of P_i in this process since the analogue, arsenate, may replace the P_i but the potent activator, sulphite, can not. It seems that the newly synthesised ATP is not extensively released from the enzyme when added ATP is the substrate nucleotide, since adenylyl imidodiphosphate inhibition only fractionally inhibits the acceleration of the decay during the first few flash-driven turnovers, although it significantly inhibits when added prior to the ATP. This would also be expected from the high phosphate potential in the external medium in the presence of added ATP.

Since the carotenoid shift decay kinetics are similar with either added ATP

(with ATPase pre-loaded with ADP) or added ADP, it seems likely that neither the release of ATP nor the binding of ADP require a significant energy input. We suppose that before the flash and with ADP as added substrate, there is a simple equilibrium binding between nucleotide and ATPase

$$E + ADP \rightleftharpoons E \cdot ADP$$

and that the concentration of nucleotide required for half-maximal effect is a reflection of this equilibrium. The specificity for nucleotide diphosphate phosphorylation is apparently governed by the binding affinity of the nucleotide for the enzyme since, at least with ADP, GDP and IDP, the kinetics of the carotenoid shift decay are similar with all three nucleotides. It seems that once nucleotide diphosphate is bound, phosphorylation proceeds regardless of the nature of the nucleotide base moiety, i.e. the specificity of the overall catalysis is determined by the binding reaction and not the phosphorylation reaction.

This reasoning does not take cognizance of the interpretations of isotopic exchange experiments with mitochondrial systems which invoke an energy-dependent binding of ADP [25] or release of tightly bound ATP [26]. Our data are not readily explained by such models but rather suggest that the energy-requiring step in catalysis is the phosphorylation reaction itself.

Acknowledgements

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