ATP-INDUCED ABSORBANCE CHANGES AROUND 515 nm FOLLOWING LIGHT-ACTIVATION OF THE LATENT ATP-HYDROLASE IN INTACT CHLOROPLASTS

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1. Introduction

Illumination of chloroplasts with strong light in the presence of thiol reagents leads to activation of the latent ATP-hydrolase [1–8]. Upon ATP-hydrolysis, reverse coupling reactions are induced (review [9]), resulting in acidification of the thylakoid interior space [8,10,11], reduction of the primary PS II acceptor Q [7–9,11] and stimulation of chlorophyll luminescence [12,13]. Applying an extrinsic field-indicating probe, oxonol VI, ATP-induced formation of a membrane potential was first observed in [14]. This was confirmed and extended in [15]. So far, however, all attempts failed to detect ATP-induced absorbance changes of the intrinsic field-indicating pigments (carotenoid shift) peaking around 515 nm [16]. As pointed out in [17], the carotenoid shift may reflect a more primary type of 'localized potential' than the 'bulk potential' sensed by oxonol VI. Main difficulties with such measurements are substantial optical disturbances caused by the stirring of the sample and upon injection of the ATP, as well as by ATP-induced light-scattering changes [7,18].

We report here on a measuring system in which stirring noise and overlapping scattering changes are sufficiently reduced to detect ATP-induced absorbance changes with satisfactory reliability. We will show that the ATP-induced difference spectrum in the blue–green region is practically identical to that of the light-induced carotenoid shift. The rapidness and amplitude of the ATP-induced response suggest that formation of a substantial membrane potential constitutes a very early step in ATP-induced reverse coupling.

Abbreviations: PS II, photosystem II; Q, primary electron acceptor of PS II

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2. Materials and methods

Intact chloroplasts were isolated from freshly harvested spinach leaves as in [19]. The chloroplasts were stored at 0°C in darkness. Intactness was routinely 80–90% as estimated by the ferricyanide method [20]. Activation of the ATP-hydrolase, hypotonic treatment and measurement of absorbance changes were carried out at 10°C with continuous stirring in the specially designed cuvette described below. Routinely, 30 μl intact chloroplast suspension (1.5 mg chl/ml) in buffer B (0.33 M Sorbitol, 40 mM Hepes/KOH at pH 6.7, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM MnCl, 2 mM EDTA and 0.5 mM KH_2PO) were diluted with 100 μl buffer C (as buffer B but at pH 7.8) and activated for 1 min by heat filtered white light (300 W/m^2) from a tungsten–halogen lamp. Immediately following light activation, the chloroplast suspension was made hypotonic by addition of 350 μl of 5 mM Hepes/KOH buffer pH 7.8 containing 5 mM $MgCl_2$ and 7% Ficoll. After 30 s, 350 μl buffer C (modified to contain 10 mM $MgCl_2$) was added, resulting in a final reaction medium of half isotonic strength and ~60 μg chl/ml. Upon closing of the cuvette with the fiberoptic adapter perspex cone, part of the suspension was squeezed out, giving a final volume of 700 μl. ATP (0.7 M, monomagnesium salt brought to pH 7.8 with KOH) was injected in 1 μl aliquots 3 min after light activation.

The absorbance measurements were carried out with a laboratory-built single-beam spectrophotometer,

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employing trifurcated fiberoptics and a specially designed stirring cuvette with the characteristics of an 'integrating sphere' to minimize apparent absorbance changes caused by light-scattering changes. The fiberoptics-cuvette asembly was essentially similar to that described for fluorescence measurements [6] except that the measuring-light fiberbundle was connected to the exit slit of a monochromator, and the magnetic stirring was with a disc mirror mounted on a disc-shaped magnet. The depth of the cuvette (from surface of sealing perspex cone to mirror surface) was 0.5 cm, resulting in an effective pathlength of 1 cm. Calibration of absorbance changes was by comparison with equivalent absorbance changes brought about by addition of known volumes of water. According to the Lambert-Beer law, $\Delta A(H_2O) = A \times \Delta V/V$. Actinic light from a tungsten/halogen lamp was filtered through 6 mm Schott RG 665 and was applied at 10 W/m^2. The photomultiplier tube was protected from actinic light and from chlorophyll fluorescence by 8 mm Corning CS 4-96 filter.

3. Results and discussion

The intact chloroplast contains all ingredients for light-activation of the latent ATP-hydrolase [21–23]. To assess ATP-hydrolase activity, the intact chloroplasts are normally exposed to hypotonic conditions after light-activation has taken place, by which treatment immediate access of added ATP is possible [21,22]. Here, we report experiments with such light-activated, hypotonically treated chloroplasts, although in principle very similar (albeit slower) responses were also observed in activated intact chloroplasts upon addition of dihydroxyacetonephosphate, for which system ATP-induced reverse coupling reactions were already observed [23].

In fig.1A the time course of the ATP-induced absorbance change at 515 nm is shown, as recorded in our laboratory-built single-beam spectrophotometer. For comparison the figure also displays the change observed at 535 nm, where one should expect pronounced scattering changes [18,24]. The ATP-induced

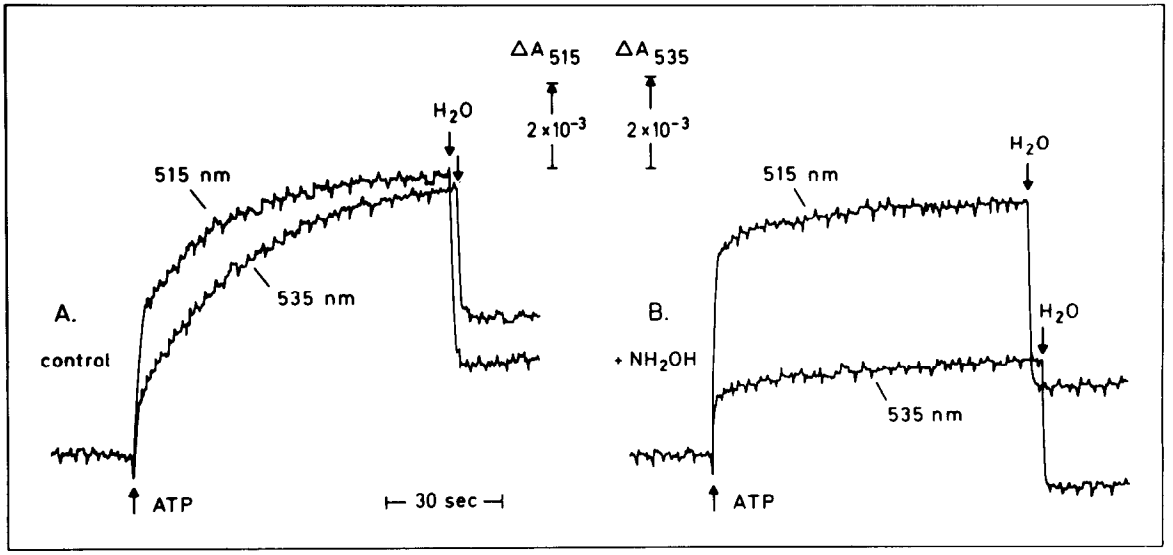

Fig.1. ATP-induced absorbance changes at 515 nm and at 535 nm. ATP was added as 1 μl aliquots of a 0.7 M solution (pH 7.8) resulting in a final concentration of 1 mM: (A) control, without addition of NH_2OH; (B) in presence of 5 mM NH_2OH, added as 3.5 μl aliquots of a 1 M solution (pH 7.8) immediately after light-activation. At the end of each run, 5 μl water were injected for calibration of the absorbance difference scale (section 2). Absolute absorbance values at 515 nm and 535 nm with the given samples were 0.62 and 0.40, respectively. The total signals at the different wavelengths were set to the same amplitude by adjusting the photomultiplier voltage. The rate of absorbance decrease produced by water-addition suggests a half-time of mixing in the order of 0.5 s.

absorbance changes at 515 nm as well as at 535 nm show biphasic rise kinetics, the total changes being about equal at the 2 wavelengths. However, the ratio of the rapid phase to the slow phase is substantially larger at 515 nm as compared to that at 535 nm. Without preceding light-activation or in presence of either an energy transfer inhibitor (Dio-9) or of an uncoupler (gramicidin), added following light-activation, the rapid as well as the slow ATP-induced absorbance changes were suppressed (not shown). Thus ATP hydrolysis initiates two separate optical changes in this wavelength region, which differ in rate. The rapid change is likely to be directly related to a carotenoid shift (peaking around 515 nm), while the slow one presumably reflects the ATP-induced increase of light-scattering [18]. Similar ATP-induced changes were observed with a commercially available spectrophotometer (Aminco DW-2), however with the signal/noise ratio being considerably lower (~1/4 at 515 nm and ~1/8 at 475 nm).

To determine a reliable difference spectrum for the rapid phase of the ATP-induced absorbance change, it was necessary to specifically suppress the slow rise component. Suppression of the slow phase was achieved by addition of substances which dissipate the transthylakoidal proton gradient in an electroneutral manner, as is the case with NH_4Cl, methylamine and nigericin. However, these substances induced a general drift of absorbance, making determination of the ATP-induced absorbance change difficult. A suitable substance, not giving rise to signal drift, is NH_2OH. When added following light-activation, 5 mM NH_2OH eliminates most of the slow rise component, while the rapid phase is somewhat enhanced. NH_2OH did not affect the ATP-induced ΔpH formation, as judged from measurements of 9-aminoacridine fluorescence quenching (not shown). NH_2OH is known to block the donor side of PS II [25]. Hence, it appears that an active PS II is required for ATP-induced scattering changes, as demonstrated for light-induced scattering changes [27].

The amplitude of the rapid ATP-induced absorbance change was determined in presence of NH_2OH in the wavelength region between 430 nm and 560 nm by using a separate, light-activated sample for each wavelength point. The resulting difference spectrum is shown in fig.2. This spectrum is very similar to the well-known light-induced carotenoid shift, with a peak at 518 nm, an isosbestic point at 491 nm and a minimum at 475 nm (which, however, appears somewhat less pronounced than that of the light-induced change; cf. fig.1 of [27]). The amplitude of the ATP-induced absorbance increase at 518 nm is, under otherwise comparable conditions, 80% of that which is induced transiently during continuous illumination with light of 10 W/m^2. Half-maximal response was induced by 2×10^{-5} M ATP similar to the value observed for ATP-induced stimulation of chlorophyll luminescence [12,13]. Saturating concentrations of ATP were applied in the experiments reported here to ensure rapid response of the system.

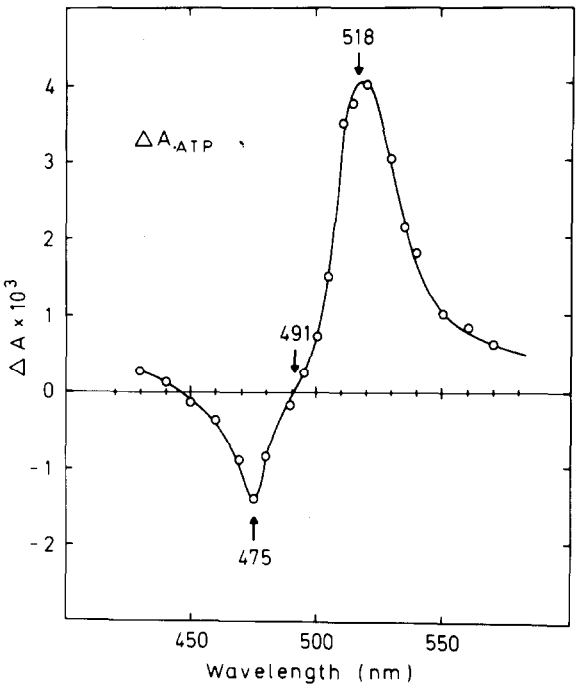

Fig.2. Difference spectrum of ATP-induced absorbance changes. For each point a fresh sample was used as described in fig.1, with 5 mM NH_2OH present. For determination of the absorbance changes, the small dilution effect caused by injection of the 1 μl ATP-solution was taken into account.

Uncouplers such as gramicidin D or desaspidin, which dissipate the ΔpH as well as the $\Delta\psi$ part of the protonmotive force, completely suppressed the ATP-induced carotenoid shift in presence of NH_2OH (not shown). Fig.3 shows the effects of sequential additions of nigericin and valinomycin on the ATP-induced 515 nm-change. Nigericin, which by acting as an electroneutral K^+/H^+ exchange ionophore will collapse only the ΔpH, did not reverse the absorbance increase. However, when valinomycin was added as well, col-

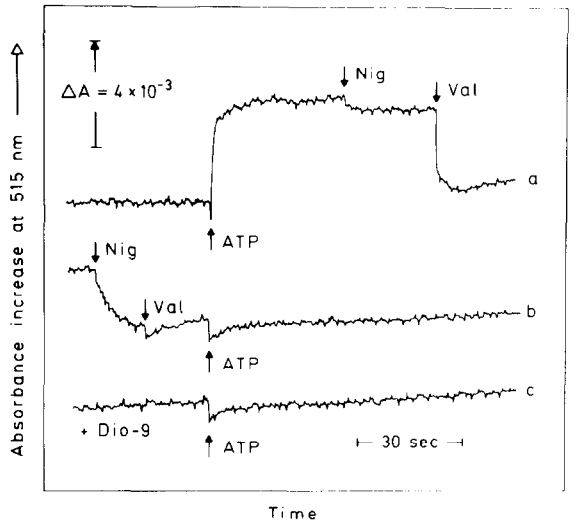

Fig.3. ATP-induced absorbance change at 515 nm in presence of NH_2OH. Effect of inhibitors: (a) consecutive addition of nigericin (0.7 μl 0.1 mM solution in methanol, final conc. 0.1 μM) and valinomycin (0.7 μl 0.1 mM solution in methanol, final conc. 0.1 μM) following the ATP-induced change; (b) addition of nigericin and valinomycin as in (a), but before ATP-addition; (c) presence of Dio-9, which was added immediately following light-activation at 2 μg/ml final conc.

lapsing also the $\Delta\psi$ via K^+-pores, the absorbance increase was abolished. Dio-9, an energy transfer inhibitor of the ATP-ase, prevented the ATP-induced absorbance change (fig.3). A similar inhibitory effect was observed with TNBT (Tri-N-butyl-tin) (not shown).

4. Conclusions

By applying a specially designed measuring system, it was possible to determine ATP-induced absorbance changes, the difference spectrum of which is very similar to that of the well-known carotenoid shift [16,27]. These spectral changes and their properties with respect to light-activation, uncouplers and ATPase inhibitors give evidence that ATP-hydrolysis produces a transthylakoidal field similar to the one induced by light. In principle, the same conclusion was reached in [14] and [15] where the extrinsic field-indicating probe oxonol VI [17] was applied. A possible advantage of the intrinsic carotenoid probe could be that it reflects a more primary type of 'localized potential' than the 'bulk potential' sensed by oxonol VI. Indeed, comparing the light-induced absorbance changes of carotenoids and oxonol VI, it was suggested [17] that the carotenoid shift monitors an intramembrane, energy conserving event prior to $\Delta\psi$ formation, while the conversion of this to a bulk $\Delta\psi$ is supposed to be reflected by the onset of the oxonol VI shift. This aspect calls for a careful comparative study of the properties of the ATP-induced carotenoid shift and the ATP-induced oxonol VI changes.

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