ON THE MECHANISM OF THE ENERGY-DEPENDENT QUENCHING OF ATEBRIN FLUORESCENCE IN ISOLATED CHLOROPLASTS

S. SCHULDINER and M. AVRON
Biochemistry Department, Weizmann Institute of Science, Rehovot, Israel

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1. Introduction

Recently, a very interesting observation relating energy generation to the quenching of the fluorescence of the uncoupler atebrin in isolated chloroplasts has been described by Kraayenhof [1]. It was shown that the required energy could be provided by electron transport, ATP hydrolysis or a pH gradient, and it was therefore suggested that the extent of quenching may be used to measure the 'energy state' of the chloroplast [1, 2]. Since several pools of energy storage can be in equilibrium with the 'energized state' (defined here as the state necessary for ATP formation) it is not clear whether the fluorescence quenching is measuring one of these pools or the state itself. One candidate may be the H^+ gradient across the membrane [3]. It was previously shown that in subchloroplast particles H^+ uptake can be abolished without affecting the rate of ATP formation [4]. We, therefore, checked in this system the relationship between fluorescence quenching and H^+ gradients.

As will be shown atebrin was found to be distributed between the inside of the chloroplast and the solution according to the ratio of proton concentration. In subchloroplast particles the quenching of atebrin fluorescence bore no relation to the capacity of the subchloroplast particles to form ATP. The quenching of the fluorescence observed seems due to several factors, among which a screening effect of the chlorophyll molecules on the exciting light is a major factor.

2. Materials and methods

Preparation of lettuce chloroplasts and measurements of proton uptake and photophosphorylation were as previously described [3, 5, 6]. Digitonin subchloroplast particles were prepared as in [7]. Fluorescence measurements were performed in an Aminco-Chance Dual wavelength spectrophotometer, with the fluorescence exciting light provided by the instrument. Emission was measured with an EMI photomultiplier with S20 sensitivity (type 9698B) through c.s. 3-71 and c.s. -4-96 Corning filters. Actinic light was provided by a 500 W projector lamp and filtered through a 680 nm Baird Atomic interference filter (30 nm half band width). Where photophosphorylation and fluorescence changes were followed simultaneously a pH electrode was introduced into the cuvette and the signals were recorded on a multichannel Rikadenki recorder.

Atebrin or methylamine uptake and osmotic volume of the chloroplast were measured essentially as previously described [8].

3. Results and discussion

Fig. 1 illustrates, as was previously shown [4, 7], that light induced proton uptake in subchloroplast particles was inhibited by concentrations of NH_4Cl which did not influence photophosphorylation. The light induced quenching of atebrin fluorescence clearly behaved parallel to the proton uptake, rather than to ATP formation. Also, as previously shown [4, 7], the extent of the proton uptake was lower than that of chloroplasts, and so was the extent of

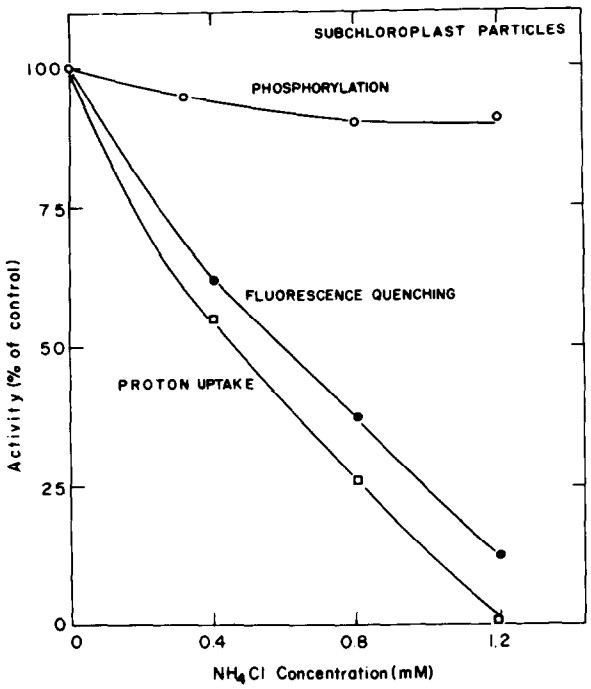

Fig. 1. Effect of NH_4Cl on ATP synthesis, proton uptake and the quenching of atebrin fluorescence in digitonin subchloroplast particles. Subchloroplast particles were prepared and the reaction carried out as described in Methods. The reaction mixture contained, in a final volume of 3 ml: 0.8 mM phosphate; 40 mM KCl; 6 μM pyocyanine; 2 mM $MgCl_2$; 0.4 mM ADP; 10 μM atebrin and subchloroplast particles containing 11 μg chlorophyll, pH 7.4. Control activities were: ATP synthesis, 140 μmoles/mg chlorophyll/hr, proton uptake, 5 nmoles/mg chlorophyll; fluorescence quenching, 80% of total atebrin fluorescence.

quenching. Thus, in these particles, as well as in chromatophores [9], a clear separation between light induced fluorescence quenching or proton uptake and the process of photophosphorylation is observed. However, it should be noted, that the decrease in the observed proton uptake does not necessarily indicate a similar decrease in the pH gradient. Indeed, the decrease in the quenching of atebrin fluorescence indicates only a partial disappearance of the pH gradient [see also 2, 10].

The nature of the correlation between proton uptake and the quenching of atebrin fluorescence was further investigated considering the possibility that atebrin, a molecule with two basic amines, may distribute across the chloroplast membrane in response to the proton concentration gradient, in a way similar to that of other weak acids or bases, such as DMO (dimethyloxazolidinedione) [11] in mitochondria, NH_3 [12] and methylamine [8] in chloroplasts. The results presented in table 1 indeed show that upon illumination atebrin was taken into the chloroplasts, and that its distribution was determined by the H^+ gradient across the membrane. Thus, the fluorescence quenching of atebrin may serve as a powerful tool for following proton gradients generated across the chloroplasts, but it seems to have no quantitative relation to the 'energized state'.

There remains the problem of why should there be quenching of the fluorescence of an atebrin molecule which has moved into the chloroplast. Three possible causes seem plausible: (a) Atebrin fluorescence decreases at the low pH which exists inside the chloroplast. Direct measurement of the dependence of atebrin fluorescence on the pH of the medium (unpublished observations) indicates that

Table 1
The distribution of atebrin and methylamine across the chloroplasts.

	Methylamine in / Methylamine out	Atebrin in / Atebrin out	ΔpH methylamine	ΔpH atebrin
Dark	7	17	0.8	0.8
Light	57	221	1.8	1.5

For experimental details see Methods. Reaction mixture as in fig. 1 except for the chlorophyll content which was 400 μg/ml and the addition of 5 mM sorbitol, 30 μM methylamine hydrochloride, 2 μCi/ml ^3H_2O and either 1 μCi/ml of ^{14}C-methylamine or 2 μCi/ml of ^{14}C-sorbitol. The ΔpH in the methylamine case was calculated as previously described [8]. The ΔpH in the atebrin case was calculated by a similar treatment [13] but taking into consideration both of the amino group ($pK_1 = 7.5$, $pK_2 = 10.1$), and assuming that only the non-charged species was freely permeant.

$$\frac{(\text{Atebrin})_{in}}{(\text{Atebrin})_{out}} = \frac{K_1(H^+)_{in} + (H^+)^2_{in}}{K_1(H^+)_{out} + (H^+)^2_{out}}$$

This assumption may not be entirely valid in the case of a complex molecule like atebrin. Some permeation of the singly charged species may exist, and could account for the somewhat lower ΔpH, as calculated from the atebrin values.

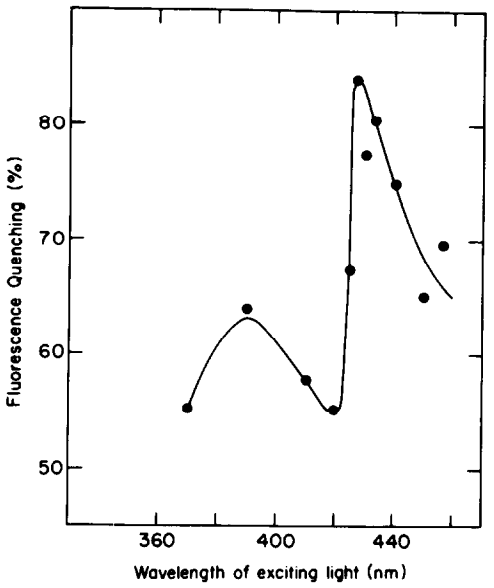

Fig. 2. Effect of wavelength of fluorescence exciting light on the extent of fluorescence quenching. Chloroplasts were prepared and the reaction carried out as described in Methods. The reaction mixture contained in a final volume of 3 ml, 10 mM Na-tricinate, pH 8.0; 40 mM KCl, 6 μM pyocyanine; 8 μM atebrin and chloroplasts containing 17 μg chlorophyll.

this effect, although present, cannot account for more than a fraction of the observed effect. (b) The inside of the chloroplast may provide a highly quenching environment due to energy transfer to chlorophyll or other components, and (c) simple screening effect whereby the membrane bound chlorophyll will absorb a portion of the incoming exciting light (normally the 436 nm line of mercury) before it reaches the atebrin molecules located inside the chloroplasts. This latter possibility was checked by changing the wavelength of the fluorescence exciting light, from one that is highly absorbed by chlorophyll to wavelengths which are not as highly absorbed. As can be seen in fig. 2, the extent of quenching varied considerably depending on the wavelength of the fluorescence exciting light around the soret peak of the chlorophyll. It is clear, therefore, that a major portion of the quenching must be attributed to such a screening effect. The actual quantitative contribution of these three effects to the observed quenching is difficult to evaluate with the present available data.

In view of the data presented, the well known uncoupling effect of atebrin [14, 15], could be explained by a mechanism similar to that proposed to explain the uncoupling ability of NH_4^+ salts [4, 12, 16]. Thus, distribution of atebrin in response to a H^+ gradient, and a leakage out of the ionised species will create a cyclic movement of protons leading to an energetically useless pumping of protons in and out of the chloroplasts [4, 16, 17]. This possibility is strengthened by the following observations: (a) Atebrin, like NH_4^+ salts, does not uncouple proton ejecting mitochondria [18] but does uncouple proton uptaking membrane fragments of *Azotobacter vinelandi* [19]. (b) The pH dependence of uncoupling by atebrin is similar to that seen with NH_4^+ salts [20]. (c) Chlorpromazin, a substance somewhat similar to atebrin, is a good uncoupler of photophosphorylation [21, 15]; chlorophenothiazine, having the same ring structure but without an alkylamine side chain, is not [22].

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