PRELIMINARY NOTE

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Oxidative phosphorylation in *Azotobacter vinelandii*. Atebrin as a fluorescent probe for the energized state

Extrinsic fluorescent probes, such as 1-anilinonaphthalene-8-sulphonic acid and ethidium bromide, have been used to study changes in the conformation of proteins[1], membranes[2,3] and sub-mitochondrial particles[3]. The binding of these probes to proteins or membranes usually leads to an increased fluorescence, and further fluorescent enhancement follows conformation changes, for example those induced by addition of GTP and NADH to glutamate dehydrogenase[1] or by energization of sub-mitochondrial particles[3,4].

A difficulty in the interpretation of such data is uncertainty in the location of the probe within the protein molecule or membrane. In this respect, the use of fluorescent compounds that interact with the system in a well-defined and specific manner, such as substrates or inhibitors of enzymes, or uncouplers of oxidative phosphorylation, offers special advantages. For example, the fluorescence of NADH is quenched when bound to glyceraldehydephosphate dehydrogenase[5]. The fluorescence of antimycin is completely quenched when it is bound to sub-mitochondrial particles or to fragments of the respiratory chain containing native cytochrome *b* (ref. 6) in the proportion of one molecule of antimycin to two molecules of cytochrome *b*. It is proposed that antimycin specifically combines with a high-energy conformation of cytochrome *b* (refs. 7, 8). KRAAYENHOF[9] has shown that the fluorescence of atebrin added to a suspension of chloroplasts is completely quenched when the chloroplasts are energized by light-induced electron transport, addition of ATP or by a pH gradient. It is interesting that, in contrast to fluorescent probes of the type of 1-anilinonaphthalene-8-sulphonic acid and ethidium bromide, the fluorescence of the specifically active probes, antimycin and atebrin, is quenched when the system is energized.

Since atebrin is an uncoupler of oxidative phosphorylation in membrane fragments from *Azotobacter vinelandii*[10], as well as of photophosphorylation, studies similar to those of KRAAYENHOF[9] have now been carried out with these fragments. Fig. 1 shows that the fluorescence of atebrin is quenched by phosphorylating particles oxidizing malate. No quenching is observed when atebrin is added to these particles when there is no oxidation, for example when added to the suspension after anaerobiosis. No quenching is observed with non-phosphorylating particles, indicating that atebrin can interact with the membrane only when the phosphorylating system is intact. This is further illustrated in Fig. 2. It is possible, as shown by PANDIT-HOVENKAMP[13], to restore phosphorylation in non-phosphorylating particles by incubation in 0.1 M KCl with a soluble protein factor. The reconstituted system has restored phosphorylating capacity[13], respiratory control[14] and a trypsin-induced ATPase activity (L. J. M. EILERMANN AND M. FEENSTRA, unpublished experiments). Fig. 2 shows that

the reconstituted system quenches the fluorescence of atebrin. Other experiments showed that the reconstituted system had no effect on the fluorescence in the absence of oxygen. The soluble factor alone also had no effect.

The almost complete quenching of the fluorescence is found only with small amounts of atebrin. Excess atebrin fluoresces with an intensity almost equal to that in the absence of particles or oxygen. The point of intersection of the two straight lines illustrated in Fig. 1 corresponds to the amount of atebrin that is bound to the

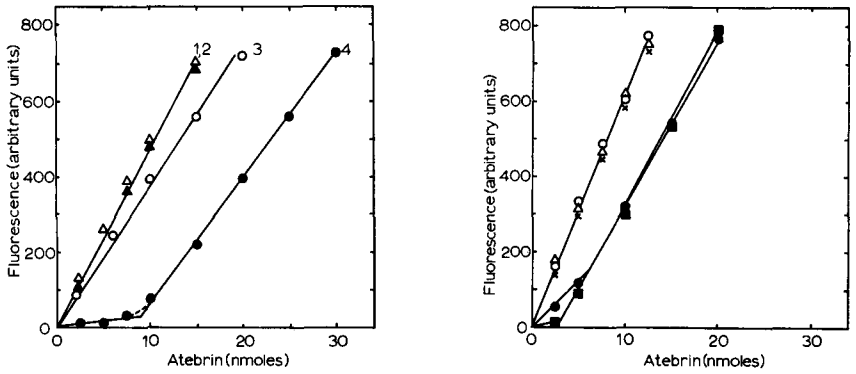

Fig. 1. Quenching of fluorescence of atebrin by energized Azotobacter particles. Various amounts of aqueous atebrin were added with a microsyringe to a suspension of particles in 30 mM phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 1500 units catalase (Boehringer) and 25 mM malate in a volume of 1.0 ml. The fluorescence was measured in the apparatus described by KRAAYENHOF[9,11], with excitation at 405–430 nm and emission viewed through a 505 nm interference filter (Vitatron). Curve 1 (△—△), non-phosphorylating particles[12] (1.6 mg protein); oxygen exhausted before additions of atebrin. Curve 2 (▲—▲), non-phosphorylating particles; sufficient H_2O_2 added before atebrin additions to keep the suspension aerobic. Curve 3 (○—○), phosphorylating particles[12] (0.25 mg protein); oxygen exhausted before addition of atebrin. Curve 4 (●—●), phosphorylating particles; sufficient H_2O_2 added before atebrin addition to keep the suspension aerobic.

Fig. 2. Quenching of fluorescence of atebrin by energized reconstituted Azotobacter particles. Conditions as in Fig. 1. ×—×, no particles; ○—○, phosphorylating particles (0.13 mg protein), anaerobic; ●—●, phosphorylating particles, aerobic; △—△, non-phosphorylating particles (0.6 mg protein), aerobic; ■—■, particles (0.27 mg), reconstituted by preincubation of 1.2 mg non-phosphorylating particles and 0.5 mg soluble factor at 0° for 45 min in 0.1 M KCl[13], aerobic.

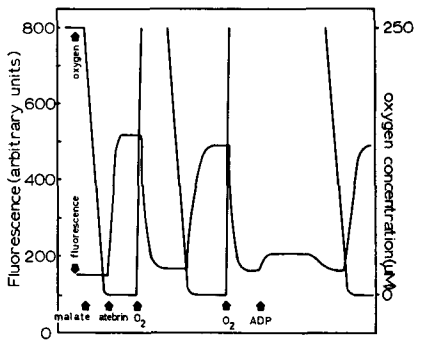

Fig. 3. Reversibility of quenching of atebrin fluorescence. Oxygen concentration and fluorescence were measured simultaneously. Additions of 25 mM malate, 15 μM atebrin, 2–4 mM H_2O_2 and 100 μM ADP to the medium of Fig. 1 containing 0.16 mg (protein) of phosphorylating particles, where shown.

particles in such a way that its fluorescence is quenched. This amount is proportional to the concentration of particles.

The quenching is reversible. When oxygen is added, the fluorescence is quenched and when the oxygen is consumed the fluorescence is restored (but not completely) to its original value (Fig. 3). The fluorescence is also restored by addition of other uncouplers or inhibitors (experiments not shown here) as found also by KRAAYENHOF[9], and also partially by ADP (Fig. 3). In this respect, it is important to note that the amount of atebrin used in these experiments (less than 50 nmoles/mg protein) is much less than that necessary for uncoupling. The P/O ratio is lowered 50% by 500 nmoles/mg protein[10].

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