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EVIDENCE FOR A SEQUESTERED SOLVENT SPACE IN THE CHLOROPLAST ATPase

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Isolated coupling factor of photophosphorylation (CF_1) covalently labeled with eosin isothiocyanate was studied by polarized laser spectroscopy. Judged by the access of oxygen bound to eosin isothiocyanate and by the librational mobility of eosin isothiocyanate we conclude that activated CF_1 encloses a volume with solvent character. In the membrane-bound enzyme the sequestered volume becomes exposed when the membrane is energized.

ATP synthesis in photosynthetically as well as in oxidatively energized membranes is mediated by a proton-translocating enzyme complex with an integral membrane portion (in chloroplasts CF_0), which acts as a proton well and a peripheral part (CF_1) which carries the catalytic activity. It is accepted that this enzyme complex converts a proton-motive force into the free energy of the ATP/ADP couple [1]. Despite intensive studies on the structure and function of the ATP synthase (see, for reviews, Refs. 2–5) the mechanistic link between proton translocation and phosphate ester bond formation still awaits elucidation. In particular, the role of drastic conformational changes of CF_1 in energy conversion and/or enzyme activation is open to question.

We studied the conformation of isolated CF_1 as function of its activation state. As described previously [6–8,11], eosin isothiocyanate, which was covalently attached to the enzyme, served as a probe for the equivalent viscosity around and at a given binding site. The results led us to postulate that the dithiothreitol-activated coupling factor CF_1 encloses a volume with solvent character (high librational mobility of inside-bound dye), which is only poorly accessible to oxygen from the bulk phase (long triplet lifetime of inside-bound dye). In the membrane-bound enzyme this sequestered domain becomes more exposed if the membrane is energized. It is conceivable that the catalytic sites for the hydrophilic substrates (ADP, ATP) are at the surface of this sequestered (aqueous?) volume.

Chloroplasts [9] and CF_1 [10] were isolated by standard procedures. CF_1 was purified as described in Ref. 6. Labeling of the purified enzyme with eosin isothiocyanate and determination of label distribution over the subunits of CF_1 were performed as previously described [11]. The Ca^{2+}-ATPase was activated by dithiothreitol (50 mM at 25°C) as described in Ref. 12 and the activity was determined as outlined in Ref 13 A suspension of the labeled enzyme in buffer medium was excited with a linearly polarized flash from a frequency-doubled, Q-switched Nd-YAG laser (JK Lasers). The decay of the photoinduced triplet state of bound eosin and the decay of the linear dichroism were measured photometrically at high time resolution as in previous work [6–8]. A rapid transient

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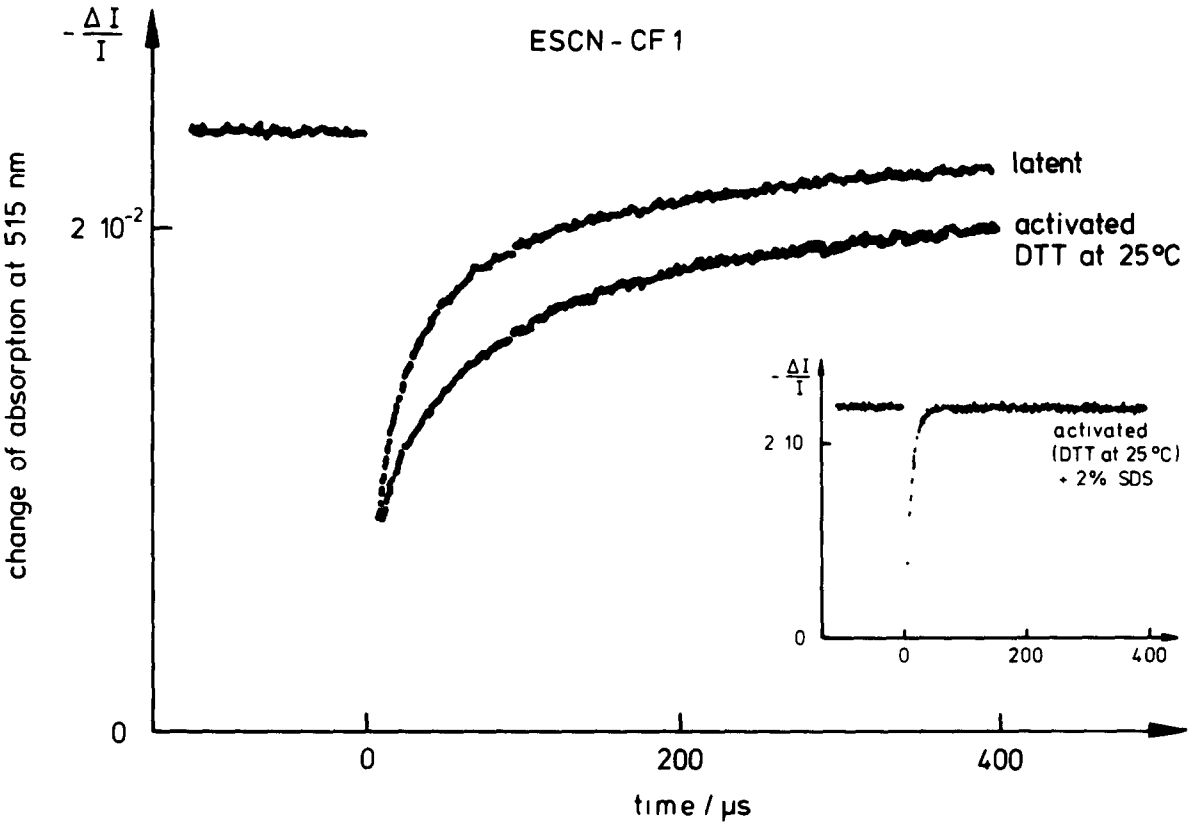

Fig 1 Change of absorption of eosin at 515 nm under excitation of isolated, labeled CF_1 with a flash from a Q-switched frequency doubled Nd-YAG laser averaging over 20 repetitions, typical laser energy, 10 mJ per flash, 10 ns duration The load of eosin isothiocyanate on CF_1 was 5 1 in all samples The measuring cell (1 ml, 0 5 cm optical path) contained bound eosin isothiocyanate, 1 μM, ATP, 5 mM, Tris-HCl, 50 mM (pH 8 0), air-saturated solution at 20°C The upper trace shows the absorption changes of latent CF_1 and the lower trace those of activity CF_1 (dithiothreitol, 50 mM, at 25°C) The inset shows the absorption change incubation of dithiothreitol activated CF_1 with 2% SDS for 10 min at 60°C Analysis of the decay for two exponentials yielded the following latent enzyme (30 μs, 78%–250 μs, 22%), dithiothreitol-activated enzyme (25 μs, 42%–130 μs, 58%) ESCN, eosin isothiocyanate, DTT, dithiothreitol

recorder (Biomation 6500) interfaced to an averaging computer (Tracor TN 1500) was used for digitizing and averaging of transient signals. Decay data were analyzed for exponentials on a TR 440 computer with the aid of a nonlinear fitting program designed to cope with noisy decay processes [14]. Time resolution was limited by the flash burst artefacts which lasted 10 μs in the documented traces at relatively low time resolution and 30 ns in the above-mentioned low-temperature experiments at high time resolution (The improvement was due to the use of a xenon flash as source of the measuring light.)

In the set of experiments which are documented in the figures the average eosin load on labeled CF_1 was 5.1 mol eosin isothiocyanate/mol CF_1, and it was distributed over the subunits as follows: α (61%), β (37%) and γ (2%). Labeling only slightly affected the ATPase activity both of the non-activated and of the dithiothreitol-activated enzyme. The respective Ca^{2+}-ATPase activities (in μmol P_i/mg protein per min) were as follows. (latent enzyme, unlabeled, 1); (latent enzyme, labeled, 0.8); (dithiothreitol, 25°C, activated, unlabeled, 7.8) and (dithiothreitol, 25°C, activated, labeled, 7.5).

A solution of labeled CF_1 was excited with a short laser flash. This populated the triplet state of

bound eosin. We monitored photometrically the population of the ground state at a wavelength of 515 or 545 nm, respectively. The transient absorption changes in Fig. 1 reflect the very rapid ground-state depletion which was caused by the laser flash and the subsequent repopulation from the triplet state in an oxygen-dependent reaction Numerical analysis of the decay revealed a rapid phase (half-decay time 25 and 30 μs, respectively) plus slower phases (250 and 130 μs, respectively) Comparison of the traces obtained with latent CF_1 and with dithiothreitol-activated CF_1 in the absence of ATP shows that activation prolonged the triplet lifetime of two eosin molecules. Relative to a total of 5.1 molecules of eosin isothiocyanate per CF_1, 1 1 had a long triplet lifetime in the latent enzyme and 3.1 in the activated one. Longer lifetime means hindered access of oxygen to the dye or deeper burying of the dye in the protein The inset in Fig. 1 shows that the five bound eosin molecules became all exposed, if CF_1 was unfolded by SDS treatment.

We investigated librational motion of bound eosin isothiocyanate relative to the protein and rotational diffusion of the protein in solution via the relaxation of the polarization anisotropy of the absorption changes in photoselection experiments,

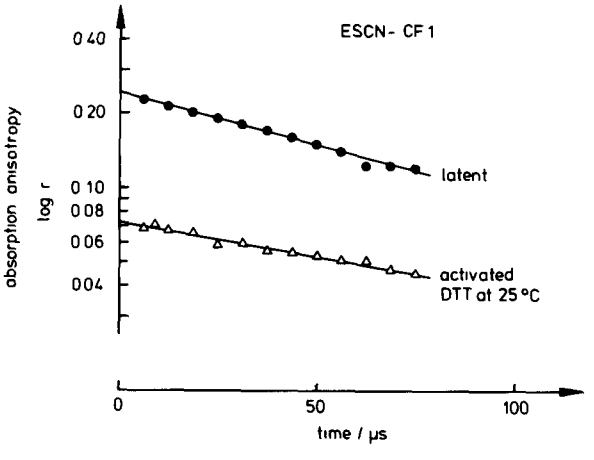

Fig 2 Time course of the absorption anisotropy (r) of eosin for latent and dithiothreitol-activated CF_1 in isotropic solution Photoselection experiments as described in Ref 6 The eosin isothiocyanate-labeled CF_1 was suspended in glycerol/water (80 20, v/v) with ATP, 1 mM, Tris-HCl, 50 mM (pH 8), at 10°C and at 2 0 P The relaxation time of the r parameter was 74 μs for the latent enzyme and 106 μs for the activated one

technically as described previously [6]. Fig. 2 shows the time course of the anisotropy in a half-log plot for labeled CF_1 in isotropic solution. Previously, we established that the theoretical maximum for the anisotropy at time zero, which is 0.4, was obtainable for immobilized eosin in our instrument (see Fig. 4 in Ref. 6). The difference between 0 4 and the actual figures at 20 μs after the flash in Fig. 2 reflected the unresolved dissipation of anisotropy by rapid librational motion of bound eosin or by resonant energy transfer. The slower decay then resulted from rotational diffusion of the protein. It is obvious that dithiothreitol activation rearranged CF_1 in a way which increased the librational mobility of or resonant energy transfer between some bound eosin isothiocyanate molecules. We asked which of the two alternatives (librational motion or resonant transfer) was dominating. For this we measured the initial value of the dichroism at moderately lowered temperature (243 K) and at very high time resolution (duration of the flash burst artefact 30 ns) With activated CF_1 (5.1 eosin isothiocyanate molecules per CF_1) we obtained an initial value of the r parameter of 0.36 which is close to the ideal 0.4 The anisotropy decayed at a relaxation time of 340 ns to the value which is shown in Fig. 2 Since the line shape of bound eosin and therewith the Forster overlap integral was not appreciably changed under these conditions (see also Ref. 23), we concluded that the lowering of the initial value of the r parameter in Fig. 2 was in fact due to librational motion and not primarily to resonant energy transfer

Taking the summation law of Weber [16] into account and under the simplifying assumption that we had only two classes of bound dye molecules, we calculated on the basis of Fig. 2 that about two eosins were highly mobile in the latent enzyme and about four in the dithiothreitol-activated one.

Another feature apparent from Fig. 2 is that the correlation time for protein rotational diffusion is longer for the activated enzyme. This implies that CF_1 became larger and/or more eccentric upon activation (see also Ref. 6).

We reconstituted eosin isothiocyanate-labeled and dithiothreitol-activated CF_1 into thylakoid membranes which were fully depleted of CF_1 by NaBr treatment [13]. Again the triplet decay of bound eosin was measured. Fig. 3 shows that in

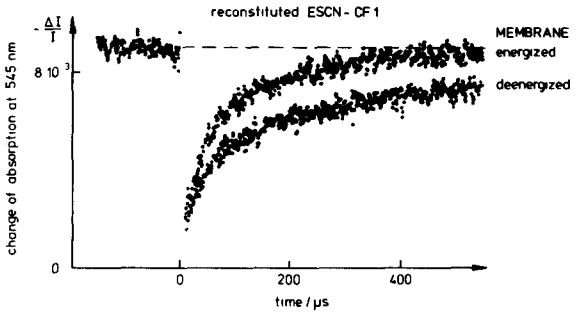

Fig 3 Change of absorption of eosin at 545 nm of a suspension of chloroplasts, which were depleted of CF_1 by NaBr treatment and later reconstituted with eosin isothiocyanate-labeled CF_1 Excitation with a pulse from a Nd-YAG laser and electronic conditions as in Fig 1 The suspension contained chlorophyll, 50 μM, eosin isothiocyanate, 0 5 μM (with 5 1 mol eosin isothiocyanate/mol CF_1), Tris-HCl (pH 8), 50 mM, ADP, 2 mM, bovine serum albumin, 1 g/l, $MgCl_2$, 5 mM, P_i, 2 mM, phonazine methosulfate, 0 3 μM in air-saturated water Side illumination (only for 'energized membrane'), 0 1 W/cm^2, was on for 200 ms and it was switched off 5 ms before the laser flash The eosin isothiocyanate-CF_1 was activated by dithiothreitol (25°C) before reconstitution For reconstitution NaBr-treated chloroplasts [13] were incubated with eosin isothiocyanate-CF_1 (at 5 g chlorophyll/g eosin isothiocyanate-CF_1) at 4°C for 10 min in the following medium Tris-HCl (pH 8), 50 mM, ATP, 1 mM, bovine serum albumin, 1 g/l, dithiothreitol, 100 μM They were centrifuged for 10 min at 10000 × g and resuspended in the above medium Analysis for two exponentials yielded the same relative extent as in Fig 2, however, different decay times latent enzyme (36 μs, 78%–188 μs, 22%) and activated enzyme (36 μs, 42%–188 μs, 58%)

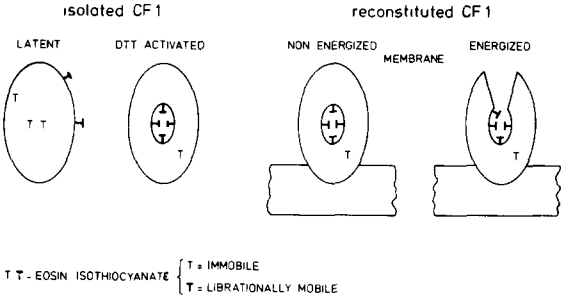

Fig 4 Speculative scheme for the location of bound eosin isothiocyanate The scheme is based on the triplet lifetime and on rotational mobility of the dye only obtained for the isolated enzyme Eosin molecules which are depicted as sequestered in the enzyme had long triplet lifetime.

the absence of a proton-motive force across the membrane (i e., in the dark or in light but plus uncoupler) the proportion of rapid to slow decay was very similar to that in the isolated enzyme. About three out of the five eosin isothiocyanate molecules were very poorly accessibly to oxygen. However, this changed to about one if the membrane was energized

The results are summarized and interpreted as follows (see Fig. 4). Isolated CF_1 was labeled with eosin isothiocyanate in a way which did not appreciably impair its ATPase activity nor its ability for reconstitution. The average load was 5.1 eosin isothiocyanate/CF_1, with three on the α- and two on the β-subunit In the latent state isolated CF_1 exposed about four of the eosin isothiocyanate binding sites to the bulk medium, while about one site was more deeply buried within the protein

Activation of the enzyme by dithiothreitol at room temperature transferred two further sites to the protein interior. However, this was, strikingly enough, accompanied by orders of magnitude higher librational mobility for at least two eosins The most likely interpretation is that activation transfers two of the eosins from the outer surface of the protein into a domain, which has solvent properties with regard to the apparent viscosity but which is deeply sequestered, so that oxygen has only poor access. It is conceivable that this domain is aqueous and that it accommodates the hydrophilic substrates ADP and ATP We also found that this domain opened partially if CF_1 was located in the energized thylakoid membrane That only some of the three sequestered eosin isothiocyanate molecules have better accessibility to oxygen (see Fig. 3) could be interpreted as follows The duty cycle of CF_1 as an ATP synthase in the fully energized membrane is such that it only opens for say one-half of the cycle period. Alternatively, it might be argued that only one-half of the enzymes were cycling (see also Ref 21).

The opening and closing of CF_1 under energization and deenergization of the thylakoid membrane was previously demonstrated by very different experimental techniques, (e.g., see Refs 7 and 17–19) Also, an internal domain of low electron density was claimed [20] In this paper we contribute the observation that solvent character has to be ascribed to a sequestered domain in the coupling factor for photophosphorylation. It is conceivable that the release of ATP from the seques-

tered domain is the main energy-requiring step as postulated by Boyer [22].

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