HEAT-ACTIVATED CONFORMATIONAL CHANGES OF ISOLATED COUPLING FACTOR OF PHOTOPHOSPHORYLATION CF_1

Studies via triplet lifetime of bound eosin-isothiocyanate

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1. Introduction

Photosynthetic formation of ATP in chloroplasts involves the coupling factor (CF_1) which is bound to thylakoid membranes. The coupling factor has been isolated and purified to homogeneity [1]. It has a Ca^{2+}-dependent latent ATPase activity which may be activated by digestion with trypsin or by heating [2].

Heat activation of isolated CF_1 causes significant conformational changes, as judged by accessibility to inhibitors [3] and, recently, from hydrogen–tritium exchange studies [4]. When CF_1 is bound to the membrane, the ATPase is activated by a protonmotive force in the presence of reducing agents [5–7]. This activation process of the enzyme is also accompanied by changes in conformation [8–11]. The current work was an attempt to see if the flash spectrophotometric technique with eosin-SCN as triplet probe, applied to the membrane bound enzyme [11], could give further insight into the characteristics of conformational changes suffered by soluble CF_1 during heat activation of its ATPase activity.

Eosin-isothiocyanate (eosin-SCN) can be covalently bound to several distinct sites in CF_1 [11–13]. Under excitation of the dye with a laser flash, a relatively stable triplet state is generated. Spectrophotometric detection of this triplet state allows to follow conformational changes of the enzyme in either of two ways:

(1) Since the triplet lifetime depends on the access of dioxygen to the given binding site, it is sensitive to the 'proximity' of this site to the bulk medium. Shortening and prolongation of the triplet lifetime reflect opening and closing of the enzyme structure.
(2) The relatively stable triplet state can be used for photoselection studies on the rotational diffusion of CF_1, which give information on the hydrodynamic size and shape of the isolated enzyme.

These techniques have been used to study the conformation of CF_1 in solution [2,3] and conformational changes of CF_1 in the thylakoid membrane [1]. They have also been applied to follow substrate-dependent conformational changes of the isolated Fd-NADP-oxidoreductase from spinach [14].

We studied conformational changes of the isolated coupling factor for photophosphorylation (CF_1) as function of temperature. The coupling factor was covalently labeled with eosin-isothiocyanate. The triplet lifetime of the bound dye was used as an indicator for the access of the quencher dioxygen from the bulk to the given site in the protein. We found: Heat treatment of isolated coupling factor exposes 3 additional binding sites for eosin-SCN, 2 of which are located on the γ-subunit of the enzyme. Upon raising of the temperature the triplet lifetime of eosin-SCN, which is bound to the γ-subunit, is shortened. This implies exposure of the γ-subunit to the bulk medium. The process is reversible. It shows a sharp temperature transition at 30°C with an activation enthalpy of 150–200 kJ/mol. This is the order of magnitude which is required to break a single disulfite bond. It is noteworthy that the temperature transition for the activation of the Ca^{2+}-ATPase activity occurs at 50°C.

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2. Materials and methods

[^{32}P] ATP was purchased from Amersham and used without further purification. ATP and dithiothreitol (DTT) were from Sigma Chemical Co. All other reagents employed were of analytical grade. Eosin-SCN was prepared according to [15] and dissolved in phosphate 0.1 M (pH 8) immediately before using. CF_1 was purified as in [16].

Labeling of purified CF_1 with eosin-SCN was performed in the following medium: tricine–NaOH, 40 mM (pH 8); EDTA, 1 mM; ATP, 20 mM; CF_1, 1–2 mg/ml and the indicated amounts of eosin-SCN. DTT, when present, was at 5 mM. After 4 min of incubation at 25°C (cold-labeling) or at 63°C (heat-labeling) the excess of non-bound eosin was trapped by the addition of glycine up to a final concentration of 0.1 M. Samples were then passed through Sephadex G-25 according to [17]. Protein concentration was determined with the Coomassie brilliant blue method [18] using bovine serum albumin as standard The eosin load on the protein was determined spectrophotometrically as in [10] and the Ca^{2+}-ATPase activity as in [17].

SDS gel electrophoresis was applied to determine the distribution of the label over the subunits [20]. To quantitate the amount on each subunit the general procedure in [21] was followed: After fixation of the gels the individual bands, as monitored by UV, were sliced and homogenized in 1 ml SDS 2%, tricine–NaOH 40 mM (pH 8), EDTA 2 mM. After 24 h at 50°C (under agitation) the samples were filtered through 0.45 μm Millipore filters and the phosphorescence of eosin-SCN was recorded at 555 nm (excitation at 520 nm) in a SLM spectrofluorimeter.

The instrumental set-up and the principles of the laser flash spectrophotometer are in [13]. The sample was contained in a thermostatted optical absorption cell (1 cm path, 1 ml vol.). Excitation was provided by a frequency doubled (wavelength 530 nm) Q-switched Nd-YAG Laser (JK-Lasers) at 30 ns duration and at 20 mJ/cm^2 pulse energy.

The photomultiplier was shielded from the scattered flash light by a special cut-off filter (Dr Hugo Anders) with 70% transmission at the measuring wavelength of 515 nm but OD 7 at the wavelength of excitation. Transients of the output voltage were digitized (Biomation 6500) and averaged (Tracor TN-1500). The triplet state of eosin-SCN was indirectly measured via the depletion of the ground state (at 515 nm).

3. Results and discussion

Fig.1 shows labeling of CF_1 and the Ca^{2+}-ATPase activity of the labeled enzyme as function of the eosin-SCN concentration in the incubation medium. It is apparent that heat makes extra binding sites available to the attack by eosin-SCN. The number of extra sites seems to be saturated at $3/CF_1$. This conforms with the 3 extra sites available in the membrane-bound CF_1 under energization of the thylakoid membrane [12]. It is also apparent that the ATPase activity is much more sensitive to labeling of the extra sites, while a load of 3 eosin-SCN which were attached

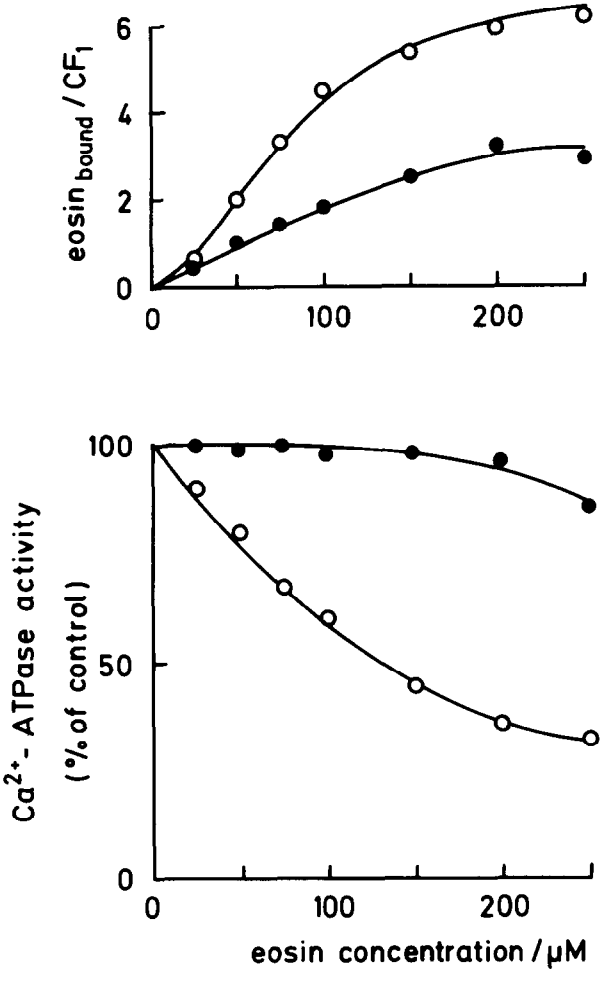

Fig.1. The amount of bound eosin-SCN on isolated CF_1 (above) and its Ca^{2+}-ATPase activity (below) as function of the eosin-SCN concentration during incubation (4 min); (○) 'heat incubation' (63°C); (●) 'cold incubation' (25°C).

Table 1
Distribution of eosin-SCN over the subunits of CF_1

Labeling mode	Eosin-SCN/ CF_1	mol Eosin-SCN/mol subunit				
		α	β	γ	δ	ϵ
'Cold'	3.3	0.72	0.70	0.50	0.03	–
'Heat'	6.4	0.90	0.85	2.30	0.12	–

Distribution of eosin-SCN over the subunits of CF_1 as function of the labeling mode ('cold' and 'heat')

in the 'cold' mode hardly affected the activity. The amount of eosin which is bound to the individual subunits of CF_1 after labeling in the 'cold' or in the 'heat' mode is documented in table 1. A slight increase in the load from 'cold' to 'heat' labeling is apparent for all subunits except for ϵ and for γ, where the load increases by almost 2. In the calculation of the eosin-SCN load we assumed a subunit stoichiometry of 2:2:1:1:2 [22]. The similarity between the labeling curves and the inhibition curves in fig.1, which were obtained under 'cold' and 'heat' labeling, with the corresponding curves, which were obtained under 'dark' and 'light' labeling of the membrane-bound enzyme [12] make a similar subunit distribution of eosin-SCN in the latter case very probable.

Fig.2 shows the time course of the absorption changes of eosin-SCN at 515 nm after excitation of the labeled CF_1 with a laser flash at $t = 0$. The time course reflects the rapid depletion of the electronic ground state and its repopulation from the triplet state. The experiment was run at 25°C in standard buffer after equilibration with air. It is apparent that the triplet lifetime is shorter in samples that were incubated in the 'cold' mode. This indicates that the 'cold' accessible binding sites for eosin-SCN are nearer to the surface of the protein. From a closer inspection of the traces in fig.2 we learn that the triplet state relaxes with a single exponential in the 'cold' incubated samples (half-decay time 15 μs) while it relaxes with two exponentials (15 μs, 67% and 170 μs, 33%) in the 'heat' incubated sample. To demonstrate that the longer triplet lifetime observed in 'heat' incubated samples is due to shielding of the respective binding sites from oxygen in the bulk solution we treated half of the 'heat' sample with SDS (2%). The result is documented in fig.3. Unfolding of the protein by this treatment transformed the slow decay phase into a rapid one. This rapid phase is also more rapid (half-time 6 μs) than the rapid phase observed in the non-unfolded protein (15 μs) both under 'cold' and under 'heat' incubation.

We studied the temperature dependence of the eosin-SCN absorption changes of 'cold' and of 'heat' labeled CF_1. The most interesting effects were observed on the slow decay component of the triplet state in 'heat' labeled samples. The result is documented in fig.4. This figure shows a half-logarithmic plot of the pseudo first-order rate constant of the triplet quenching over the inverse temperature.

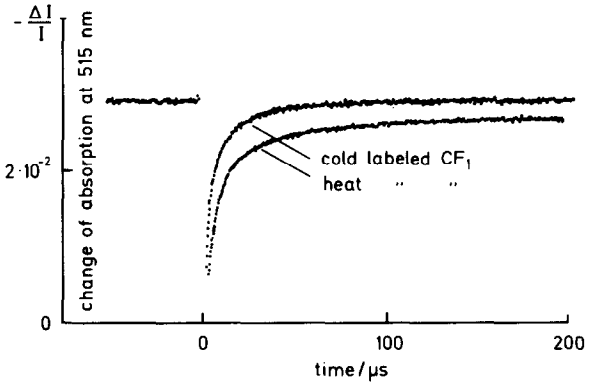

Fig.2. Time course of the absorption changes of eosin at 515 nm on isolated CF_1 which was labeled with eosin-SCN in the 'cold' and in the 'heat' mode, respectively (fig.1). The eosin-SCN concentration in both samples was adjusted to 5 μM in both samples. The eosin-SCN load per CF_1 molecule, however, was different: 3.2 in the 'cold'-labeled samples and 6.4 in the 'heat'-labeled ones.

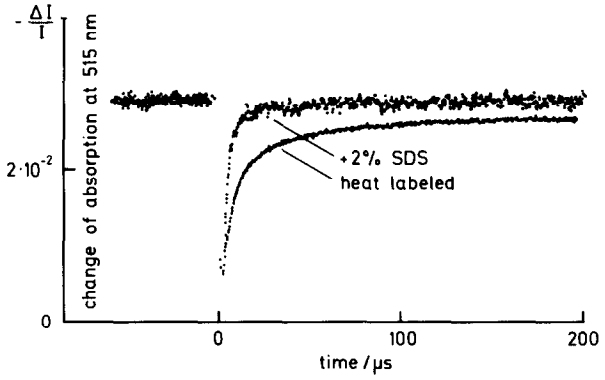

Fig.3. Time course of the absorption changes of eosin at 515 nm on isolated CF_1 which was labeled with eosin-SCN in the 'heat' mode. Lower trace shows 'heat'-labeled sample and upper trace the same sample, however after treatment with 4% SDS.

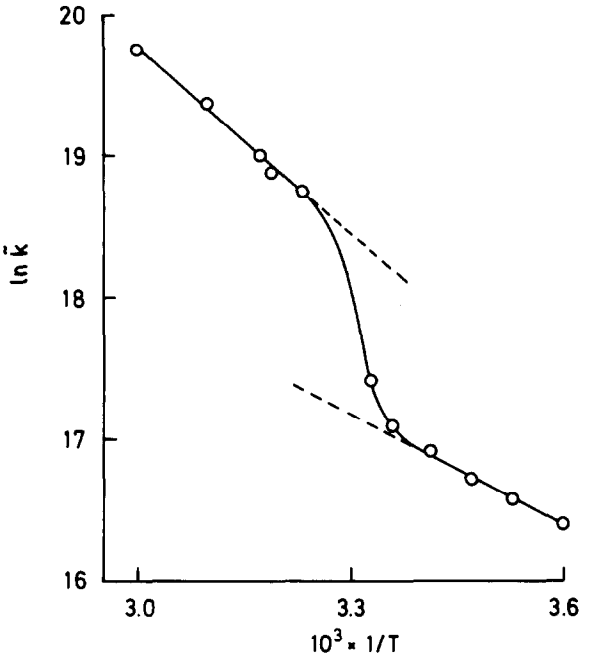

Fig.4. Arrhenius plot of the pseudo first-order rate constant of triplet quenching for those eosin-SCN molecules which are bound to those sites in CF_1 which become accessible only after heat treatment of CF_1 and which are particularly well shielded against dioxygen (half-time of triplet decay at 25°C 170 μs).

While the behaviour follows Arrhenius' law above and below ~30°C there is a sharp transition in between. It is noteworthy that the observed transition is fully reversible. A quantitative evaluation of the temperature dependence in terms of absolute rate theory yields the following: The access of dioxygen to the (heat-labeled) binding sites of eosin-SCN is limited by an activation barrier with a standard enthalpy of activation of 23 kJ/mol over 5–20°C and of 38 kJ/mol over 40–60°C. At ~30°C there is a sharp increase of the standard entropy of activation by 8.8 EU in the direction to the higher temperatures. Loosely speaking higher temperature opens more pathways for dioxygen to the eosin-SCN label, however, these have higher activation energies. The sharpness of the transition indicates an activation enthalpy of 150–200 kJ/mol. This is the order of magnitude which is required to break one disulfite bond. This behaviour was only observed for those eosins which are particularly well shielded from oxygen at labeling sites (probably on the γ-subunit) which are only accessible under 'heat' incubation. We also studied the temperature dependence of the triplet lifetime at the other sites. It did not reveal a phase transition, but a monophasic Arrhenius behaviour with an activation enthalpy of 42 kJ/mol. It is noteworthy that the Ca^{2+}-ATPase activity of labeled CF_1 showed the usual transition at 50°C.

We present evidence that heat treatment of isolated CF_1 exposes additional binding sites (predominantly $R–NH_2$-groups) for the attack by eosin-SCN. Two out of 3 such extra sites are located on the γ-subunit of CF_1. When bound to these sites, eosin-SCN is better protected from oxygen than at the 'cold' accessible sites, after the enzyme has been reclosed at lower temperature (say 25°C). It becomes more exposed again, when the enzyme is subjected to high temperature. In the intermediate temperature range around 30°C there is a sharp transition. More pathways for oxygen access are opened (at higher activation enthalphy). This transition is fully reversible. These events can be visualized as a temperature-dependent exposure of the γ-subunit to the bulk medium.

These findings are in line with reports on temperature-induced conformational changes of CF_1 as evident via inhibitors [3] and via tritium exchange [4]. They are also in line with studies on the modification of the γ-subunit with *ortho*-iodobenzoate which also required heat activation [3].

The activation enthalpy for the opening of the protein structure around the γ-subunit as taken from the transition region in fig.4 (150–200 kJ/mol) agrees well with the figure reported in [15] for the heat activation of the latent Ca^{2+}-ATPase activity of CF_1. It is close to the enthalphy which is required to break one disulfite bond. It is noteworthy, however, that the transition temperature for the activation of the ATPase (50°C, according to [4,21]) and for the exposure of the γ-subunit (30°C, this work) differ significantly. We propose that there are 2 different disulfite bonds, which have to be broken, to expose the γ-subunit, on one hand, and to activate the Ca^{2+}-ATPase on the other.

Comparing the results obtained under 'cold' and under 'heat' incubation of CF_1 with eosin-SCN with those under 'light' and 'dark' incubation of the membrane-bound enzyme [11,12] we obtain the following:

(1) Common to both treatments is an extra-binding capacity for 3 eosin-SCN/CF_1 under 'heat' and under 'light'. In the deactivated mode ('cold' and 'dark', respectively) of the enzyme, eosin-SCN,

which is bound to these sites, is highly shielded against the attack of dioxygen. It becomes exposed again after the respective activation of CF_1.

(2) The binding of eosin-SCN to sites which are accessible in the deactivated mode of the enzyme ('cold' and 'dark', respectively) is not inhibitory to any of the activities. In contrast to this, binding of eosin-SCN to the 'heat'-accessible sites inhibits the Ca^{2+}-ATPase, and binding to sites which are accessible in the 'light' mode of the membrane bound CF_1 (i.e., in the presence of a protonmotive force) inhibits the Ca^{2+}-ATPase as well as the Mg^{2+}-ATPase and the ATP-synthase activity. We presented the 'light–dark' data in [12].

(3) Two different types of inhibitions were observed: Most sensitive was the Mg^{2+}-ATPase under 'light' labeling of CF_1. Here, one eosin-SCN bound to the 'light' accessible site caused 50% inhibition. In contrast to this, inhibition of the Ca^{2+}-ATPase activity (50%) required ~2.5 eosin-SCN under both incubation modes ('light' and 'heat').

These results can be merged into the following tentative picture for the function of CF_1. The γ-subunit is one of the catalytic sites of CF_1 (ATP-synthesis, Mg^{2+}-ATPase). Since no specific interaction with P_i or with ADP and ATP has been reported, it most probably interacts with the other substrate, the proton. As it becomes exposed to the outer bulk medium when the enzyme is activated, the γ-subunit most probably serves as the gate for the passage of protons through the enzyme complex ($CF_1 + CF_0$). The site of the gate is probably also the site for the transduction of the proton-motive force into other forms of the useful work. While modification of the γ-subunit aborts ATP-synthesis, the Ca^{2+}-ATPase is less sensitive. It is probable that modification of the γ-subunit only indirectly (allosterically) affects the catalytic sites for the processing of nucleotides on the α- and β-subunits.

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