CONFORMATIONAL CHANGES OF THE ISOLATED FERREDOXIN-NADP-OXIDOREDUCTASE UPON NUCLEOTIDE BINDING AS REVEALED BY THE TRIPLET LIFETIME OF BOUND EOSIN-SCN

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1. Introduction

The existence in chloroplasts of a flavoprotein with NADPH-specific diaphorase activity was first reported by Avron and Jagendorf [1] and later it was discovered that this enzyme is essential for the photoreduction of $NADP^+$ [2]. It is active when isolated in monomeric form at M_r 35 000–40 000 [3]. In the thylakoid membrane it is accessible to impermeant antibodies and probably located at the outer side of the non-stacked parts of the membrane [4,5]. Recently, regulation of the reductase activity by pH and by light, via a transmembrane pH-difference, was demonstrated [6,7]. In vitro the reductase forms an equimolar complex with $NADP^+$ which differs from the uncomplexed form in its visible absorption spectrum [8], fluorescence yield of the flavin moeity [9] and in sensitivity to SH-reagents [10]. These alterations have been ascribed to conformational changes of the protein, without further specification of their nature and their physiological role. It is known, however, that the complex formation requires unmodified lysyl [11], arginyl [12] and carboxyl groups [7].

We attempted to further characterize the conformational changes with the probe eosin-isothiocyanate. We have applied eosin-SCN to study conformational changes of the membrane-bound [13] as well as of the isolated coupling factor of photophosphorylation [19,22]. When excited with a short laser flash eosin is efficiently transferred into a relatively stable triplet state. When iosin-SCN is covalently bound to an enzyme its triplet lifetime depends on the access of oxygen to the respective binding site. This can vary under conformational changes [13], shorter lifetime is a qualitative indicator for closer proximity of a site to the surface of a protein. Besides via its triplet lifetime we also used eosin-SCN for photoselection studies on the rotational diffusion of the coupling factor (CFI) in the thylakoid membrane. The rotational correlation time was also dependent, e.g., on the existence of a protonmotive force across the membrane and on ATP, served as another indicator of conformational changes [13].

In this study we labelled the isolated and purified ferredoxin-NADP reductase with eosin-isothiocyanate and measured the activity of the enzyme and the triplet lifetime of the probe. We found at least two binding sites which differ in their effect on the activity and in their triplet lifetime. Binding of eosin-SCN to site A inhibits the NADPH-diaphorase activity. The triplet lifetime is rather short, and therefore site A will be close to the surface of the protein. In contrast to this, binding of eosin-SCN to site B does not affect the diaphorase activity but rather the interaction with ferredoxin. The triplet lifetime of eosin-SCN at site B is longer than at A; however, it is shortened upon binding of NADPH to the (unmodified) site A. This may indicate that the environment of the ferredoxin binding site of the reductase is modified by the presence of NADPH at the other catalytic site.

2. Materials and methods

Purified ferredoxin-NADP-reductase [14] and eosin-isothiocyanate [15] were prepared according to published procedures. The enzyme was labeled with eosin-SCN (concentration in legends) by suspending it in a buffer medium which besides eosin-SCN contained:

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tricine, 50 mM (pH 8); sucrose, 250 mM; $MgCl_2$, 5 mM. After incubation (duration, see legends) the samples were passed through a Sephadex G-25 column according to [16] to remove the non-covalently bound portion of the dye. Protein was determined with the Coomassie brillant blue method [17] with bovine serum albumin as standard. The eosin load on the protein was determined spectrophotometrically as in [13].

Diaphorase activity was measured as in [18], however, 2,6-dichlorophenoleindophenole (33 μM) was used instead of ferricyanide as electron acceptor. All chemicals were of analytical grade.

The principle and the components of the laser flash spectrophotometer were as detailed in [19]. The sample was excited with a flash from a Q-switched Nd-YAG laser (JK-Lasers) at 10 ns duration (FWHM) and at 20 mJ/cm^2 pulse energy. This was not saturating at the given enzyme (5 μM) and eosin concentration in the 1 cm optical absorption cell. Absorption changes of eosin were recorded at a wavelength of 512 nm. They reflect the depletion and repopulation of the ground state of the dye. Transients of the output voltage of the photomultiplier were digitized (Biomation Transient Recorder 6500) and averaged (Tracor TN 1500). All experiments were performed at an ambient temperature of 21°C.

3. Results and discussion

3.1. *Labeling and enzyme acitivy*

Incubation of the purified ferredoxin-NADP-reductase with eosin-SCN resulted in a loss of enzymatic activity in a time- and concentration-dependent manner. Fig.1 shows the diaphorase activity as function of the eosin-SCN load on the enzyme under two different incubation conditions: at first in the presence of NADP$^+$ and then in its absence during incubation. It is obvious that NADP$^+$ protects the enzyme from

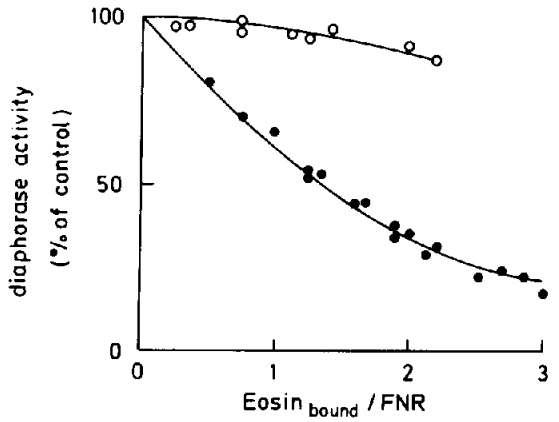

Fig.1. The diaphorase activity of the isolated and purified ferredoxin-NADP-oxidoreductase as function of the amount of eosin-SCN which is bound to the enzyme. The incubation with eosin-SCN occurred under two conditions: with (5 mM) and without NADP$^+$ in the incubation medium. The eosin-SCN load on the protein was changed under variation of the eosin-SCN concentration (up to 500 μM) and of the incubation time (up to 15 min). The control activity was 30 μequiv . mg protein^{-1} . min^{-1}. (○) Modification in the presence of NADP; (●) modification without NADP.

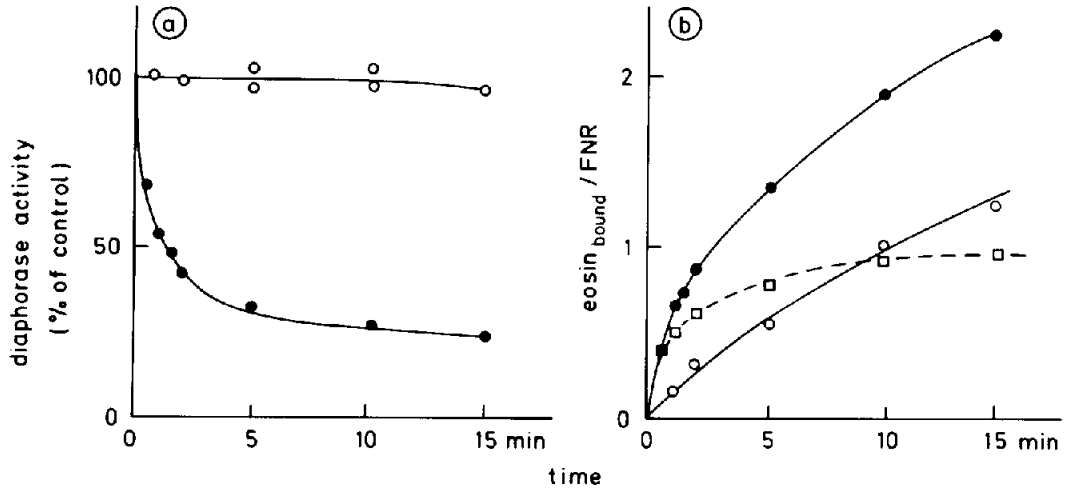

Fig.2. Diaphorase activity (a) and eosin-SCN load (b) of the oxidoreductase as function of the incubation time with eosin-SCN (170 μM). The incubation occurred under two conditions: with (5 mM) and without NADP$^+$ in the medium. (○) Modification in the presence of NADP; (●) modification without NADP; (□) difference of binding.

loss of activity. Similar results were obtained with NADPH.

Loss of diaphorase activity and the binding of eosin-SCN are plotted as function of the incubation time at a given eosin-SCN concentration (170 μM) in fig.2. It is apparent from fig.2b that the presence of $NADP^+$ during incubation protects a site with a capacity to accomodate 1 eosin-SCN/reductase. In the following we name the protected site 'A'. Comparison with fig.2a shows that the rapid binding of eosin-SCN to site A (half-rise time <1 min) in the absence of the protectant is paralleled by the loss in diaphorase activity. Fig.2b also shows that there are further binding sites, which we name 'B', 'C' and . . . in the following. We note that the inhibitory binding site is also the most reactive one. It is known that eosin-SCN reacts preferentially with amino groups at physiological pH [20,21]. It may be speculated that the loss of diaphorase activity is caused by modification of a lysine residue in the nucleotide binding domain, perhaps of the one essential lysine in [11,23].

3.2. *Laser flash spectrophotometry with the labeled reductase*

Fig.3 shows the time course of the absorption changes at 512 nm after excitation of eosin-SCN labeled reductase with a short laser flash at $t = 0$. The standard buffer medium was equilibrated with air and the mixture contained no NADP. Fig.3 shows that the triplet lifetime of eosin-SCN, when bound to the reductase, depends on the presence of $NADP^+$ in the incubation medium. In the light of the results shown in fig.1 and 2 we conclude: Binding of eosin-SCN to site A gives much shorter lifetimes of the triplet state than binding to sites B and C. [We also observed that the lifetime increased with decreased oxygen pressure (not shown).] This implies that site A, which is the site near to the NADP-binding site, is closer to the surface of the protein than sites B and C. The difference in the accessibility to oxygen is drastic, the triplet lifetimes differ by one order of magnitude (10 μs and 100 μs).

Eosin binding to other sites than A hardly affected the diaphorase activity and led to relatively long triplet lifetime. We studied the dependence of this lifetime on the binding of $NADP^+$ to the enzyme. Fig.4 shows the absorption changes of eosin-SCN bound to the reductase in the absence and in the presence of $NADP^+$ (40 μM). It is apparent that the binding of $NADP^+$ in the vicinity of the unmodified site A accellerates the access of oxygen to eosin-SCN bound to site B. In other words, binding of $NADP^+$ to one site

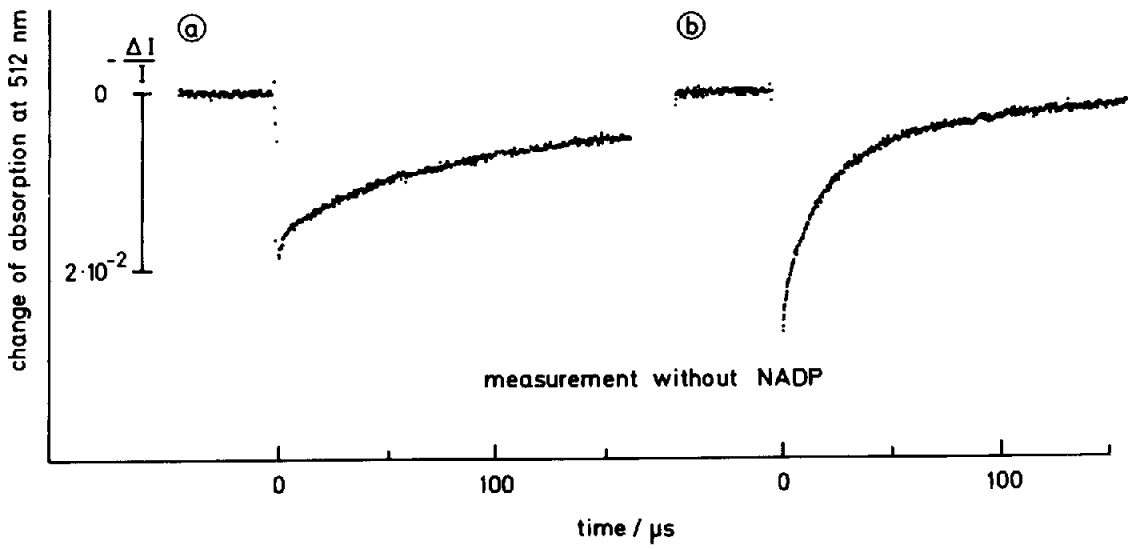

Fig.3. Change of absorption at 512 nm under excitation of eosin-SCN labeled oxidoreductase with a short flash from a Nd-YAG laser at $t = 0$. Time-per-address setting of the transient recorder: 200 ns, averaging over 30 repetitions. No $NADP^+$ in the solution during the spectrophotometric experiments. (a) Incubation of the enzyme with eosin (200 μM) for 5 min in the presence of $NADP^+$ (5 mM). Eosin-SCN load: 1.87. (b) Incubation of the enzyme with eosin-SCN (200 μM) for 5 min in the absence of $NADP^+$. Eosin-SCN load: 2.86. Enzyme concentration in (a) and (b): 5 μM.

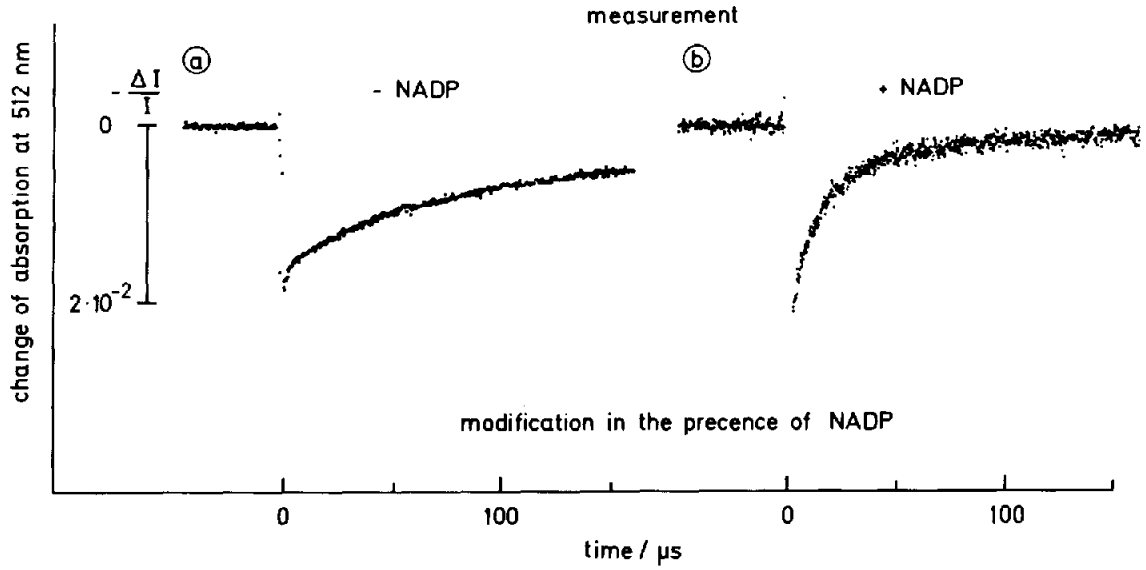

Fig.4. Change of absorption at 512 nm under excitation of eosin-SCN labeled oxidoreductase with a short flash from a Nd-YAG laser at $t = 0$. Time-per-address setting of the transient recorder: 200 ns, averaging over 30 repetitions in (a) and over 15 repetitions in (b). For (a) and (b) the incubation was the same: with eosin-SCN (500 μM) and with $NADP^+$ (5 mM) for 5 min. (a) Absorption changes recorded in the absence of $NADP^+$ and (b) absorption changes recorded in the presence of $NADP^+$ (40 μM). Eosin-SCN load: 1.87 and 5 μM enzyme in the optical cell.

affects allosterically the conformation of the protein around another site. We visualize this conformational change as an exposure of site B, which is buried within the core of the protein in the absence of $NADP^+$, to the surface.

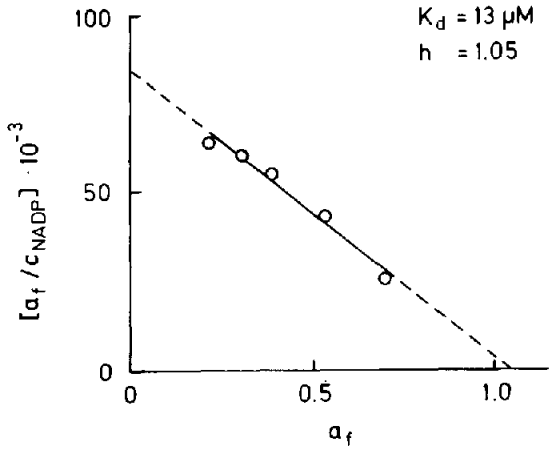

Fig.5. Scatchard plot of the relative amplitude of the rapidly decaying component in flash induced absorption changes of the eosin-SCN labeled oxidoreductase from experiments as documented in fig.4b. The relative amount of eosin-SCN with a rapid decay (10 μs *vs* 100 μs) of the laser-induced triplet state, depends on the $NADP^+$ concentration in the optical absorption cell with an apparent dissociation constant of 13 μM at an apparent stoichiometry of $h = 1.05$.

The acceleration of the triplet lifetime of eosin-SCN at site B by the binding of $NADP^+$ to a site near A was reversible (not shown). A closer inspection of the decay kinetics in fig.4b reveals two phases, one at a decay time of 10 μs the other one at 100 μs, as predominant in the absence of $NADP^+$ (see fig.4a). We varied the $NADP^+$ concentration and analysed the relative amplitudes of these phases. The result is shown in fig.5 in the format of a Scatchard plot. The appearence of the fast phase (a_f = relative amplitude of the fast phase) is related to the $NADP^+$ concentration via a nearly perfect Michaelis-Menten behaviour with $K_d = 13$ μM. This is in the range of literature figures for the interaction of the unmodified reductase with $NADP^+$ [8,11,12]. The deviation from the ideal behaviour is very low as evident from an apparent stoichiometry $h = 1.05$.

After establishment of the surface location and the vicinity to the NADP-binding site of the eosin-SCN binding site A we asked for the functional location of the eosin-SCN binding site B. We observed (not shown).

(i) Ferredoxin increases the triplet lifetime of eosin-SCN at site B by a factor of two.

(ii) Modification of site B by eosin-SCN decreases the rate of ferredoxin reduction mediated by the enzyme.

This is schematically illustrated in fig.6.

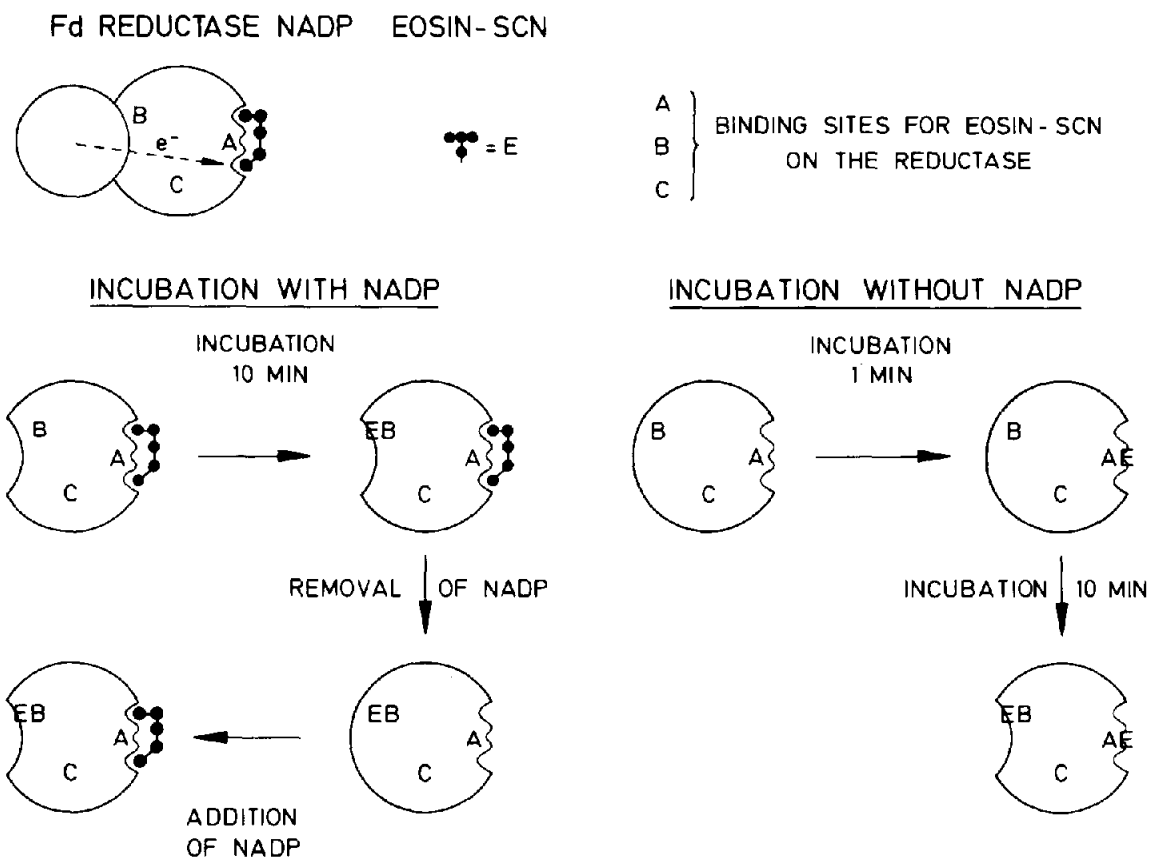

Fig.6. Schematic representation of the interpretation of the experimental data (see text).

4. Conclusions

The interpretation of the results described in this letter is summarized in fig.6. The graphs represent the ferredoxin-NADP-oxidoreductase, ferredoxin, NADP and the triplet probe, eosin-isothiocyanate. A–C denote binding sites for the probe. The triplet lifetime of the probe, which depends on the accessibility of the respective site to the quencher oxygen, places a given site deeper into the core or nearer to the surface of the protein. Our experiments lead us to the following conclusions:

1. There are at least two sites for eosin-SCN binding, A and B.
2. A is in close vicinity of the binding site for NADP, it is also very close to the surface of the protein (decay time of the triplet state 10 μs at A as compared with ~1 μs for eosin-SCN in buffer medium).
3. B is nearer to the site of the reductase, where it interacts with ferredoxin. The vicinity of the site B to the surface of the protein is allosterically regulated by the presence of NADP at a site near to A.

It is tempting to speculate that the binding of NADP to the oxidoreductase regulates the interaction of the enzyme with ferredoxin. This is under further study.

Further work with eosin-SCN labeled oxidoreductase has two interesting prospectives:

(i) The membrane-bound enzyme is probably regulated by a transmembrane protonmotive force. The eosin-SCN probe may reveal whether (and how) conformational changes are induced.

(ii) Previous studies on the rotational diffusion of the coupling factor for photophosphorylation in the thylakoid membrane revealed an extremely high microviscosity and an influence of the protonmotive force on the rotational correlation time [13].

It will be interesting to learn about the microviscosity which is seen by the oxidoreductase, in particular, as this enzyme is very probably located in the same, non-stacked portion of thylakoids.

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