Light-dependent de-activation/re-activation of *Anabaena variabilis* ferredoxin:NADP⁺ reductase

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The activity of ferredoxin:NADP⁺ reductase (FNR) was found to decline to ~20% maximal levels with little or no loss in enzyme levels when cultures of the cyanobacterium *Anabaena variabilis* were maintained in the stationary phase of growth. Re-activation of enzyme activity occurred when cells were diluted into either fresh or re-utilized media and illuminated. This reversible de-activation/re-activation process was found, *in vivo*, to be dependent on the intensity of light illuminating the cells. The de-activated form of FNR was purified to homogeneity and exhibited the same molecular mass, isoelectric-focusing pattern and *N*-terminal amino acid sequence as the native form. Both de-activated and native FNR preparations each exhibited three reactive thiol groups on denaturation in urea; however, the rate of reaction with Ellman's reagent was much faster with the de-activated form than with the native form. Both preparations contain a single disulphide bond. Upon reduction of the disulphide bond in either form of the enzyme, the five reactive thiol groups exhibited identical reactivities in the presence of urea. Steady-state kinetic analysis of the de-activated form showed a marked increase in K_m values for NADPH in diaphorase assays and an increase in K_m for ferredoxin in the ferredoxin-mediated reduction of cytochrome *c*. No significant difference in k_{cat} was observed in comparison of the de-activated with the native form in any of the above assays; however, the de-activated form did exhibit a lower k_{cat} value in the transhydrogenase assay. The de-activated form of FNR bound ferredoxin with a 16-fold lower affinity than the native enzyme. These data suggest that the de-activation of FNR *in vivo* in response to low light intensity involves an alteration in protein structure, possibly via an intramolecular thiol disulphide interchange, which influences the interaction of the enzyme with its substrates.

INTRODUCTION

Ferredoxin:NADP⁺ reductase (FNR, EC 1.1.18.11) functions in a key role in photosynthetic organisms in the transfer of reducing equivalents from Photosystem I to NADP⁺. The enzyme is located in the external portion of the thylakoid membrane [1,2], where it forms a stable electrostatic complex with ferredoxin [3,4]. Binding of FNR to the membrane is mediated by an intrinsic polypeptide of 17 kDa that is referred to as the 'FNR-binding peptide' [5]. It was reported almost 15 years ago that binding of FNR to the thylakoid membrane enhances its catalytic activity [6], and the investigation of that interaction has been carried out more recently by Vallejos' group [7]. These workers have found that illumination of the chloroplasts differentially affects the soluble and bound forms of FNR as regards their respective substrate affinities and catalytic activities, which have been interpreted to represent a possible regulatory mechanism [7]. Other authors have described photomodulation of photosynthetic electron transport involving FNR [8], as well as changes in FNR activity, as representing a possible photoregulatory process [9,10]. On consideration of the crucial function of FNR in the production of reduced nicotinamide nucleotide for cellular metabolism, it would seem reasonable to expect that its activity would be regulated in response to the light intensity to which the organism is subjected.

To probe this possible regulation of FNR activity in more detail, we have investigated the system in the cyanobacterium *Anabaena variabilis*. FNR preparations from this class of organisms have been studied by several groups [11,12] and have

been shown to be quite similar to the spinach (*Spinacia oleracea*) enzyme. No FNR-binding protein has been described for prokaryotic organisms. Owing to the ease in manipulating growth conditions of this organism and the future possibility of its genetic manipulation, we considered *A. variabilis* to be an appropriate system to investigate the influence of light on the activity of FNR and its relation to the regulation of photosynthetic electron transport. The results presented here demonstrate the existence of a reversible de-activation/re-activation of *A. variabilis* FNR *in vivo* that is dependent on the light intensity incident on the cell. Some of the molecular and catalytic properties of purified preparations of the active and de-activated forms of FNR are compared.

MATERIALS AND METHODS

Growth of cultures of *Anabaena variabilis* 1403.4b

Cell cultures were grown in the culture medium described previously [12]. Native active FNR was obtained from 10-litre cultures of cells that had been illuminated with lamps giving an intensity of 3500–4000 lx and continuously purged with air/CO₂ (19:1). After 7 days of incubation under the above conditions at 25–30 °C, cells were harvested by centrifugation and the cell paste stored at –20 °C before further processing. To induce de-activation of FNR *in vivo*, two different procedures were used: (1) after reaching the stationary phase of growth, cells were incubated in the dark with continuous purging with air/CO₂ (19:1) for 48 h before harvesting; (2) cells were grown under

Abbreviations used: FNR, ferredoxin:NADP⁺ reductase; DCPIP, 2,6-dichlorophenol-indophenol; Nbs₂, 5,5'-dithiobis-(2-nitrobenzoate); CCA, catalytic-centre activity.

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limiting light intensity (below 2400 lx) under the same conditions as described above for normal growth.

FNR purification and properties

Both active and de-activated FNR preparations were purified using the procedure previously described [12]. Preparations of enzyme exhibited A_{274}/A_{458} ratios lower than 8.0 and exhibited a single band when subjected to SDS/PAGE and stained with Coomassie Blue. *Anabaena* ferredoxin [13] and flavodoxin [14] were purified by using the procedures cited. Protein concentrations were determined either using the Lowry procedure [15] or, for purified preparations of FNR, by the absorption of the bound FAD coenzyme (ϵ_{458} 9.8 $\text{mm}^{-1} \cdot \text{cm}^{-1}$) [12]. NADPH:2,6-dichlorophenol-indophenol (DCPIP) oxidoreductase and NADPH:cytochrome *c* oxidoreductase activities were determined as described by Sancho *et al.* [12], and transhydrogenase activity was measured as described in [16]. Chlorophyll concentrations were determined as described by Mackinney [17]. Phosphorus content was determined on the basis of the spectrophotometrically quantified FNR by using the Bartlett procedure [18]. The free-thiol contents, on the basis of the spectrophotometrically quantified FNR preparations, were determined by using a 50-fold molar excess of Ellman's reagent, 5,5'-dithiobis-(2-nitrobenzoate) (Nbs₂) [19]. The total thiol contents of FNR preparations were determined in the presence of 8 M-urea and excess NaBH_4 as described by Cavallini *et al.* [20]. Binding constants for the association of either flavodoxin or ferredoxin with FNR preparations were determined spectrophotometrically as described previously [3]. *N*-Terminal sequence analysis was performed by the Emory University Microchemical Facility using Applied Biosystems equipment. C.d. spectral data were measured using an Aviv 60 DS instrument. All reagents and chemicals used in this study were of the highest quality that could be obtained commercially.

RESULTS

Our initial observation of an alteration of FNR activity (as measured by NADPH:DCPIP oxidoreductase activity) with growth conditions of *Anabaena* was the finding of only 15–20 % activity in crude cell extracts of cells harvested after 7–10 days in the stationary phase (12–15 days after inoculation) as compared with the activity in cell extracts of cells harvested during the exponential phase. The decrease in specific activity was the same whether expressed relative to total or soluble protein, total chlorophyll content or to total wet or dry weight of the cells. This decrease in activity is not due to degradation of the protein, since approximately the same amount of FNR (90 %) was isolated from late-stationary-phase cells as from cells harvested in the

Fig. 1. Time course for the dark de-activation of FNR activity *in vivo*

A culture of *A. variabilis* in the exponential phase of growth was covered with aluminum foil to block light from reaching the cells (○). A control culture was maintained under the same conditions but with constant illumination (●). At the times indicated, aliquots were withdrawn from each culture, crude cell extracts prepared, and FNR activity determined in the NADPH:DCPIP oxidoreductase assay. Specific activity is expressed in unit/mg of total protein.

exponential phase (Table 1). Rocket immunoelectrophoresis (using rabbit antisera prepared against *Anabaena* FNR) of crude cell extracts from both cell growths were used to quantify the amount of FNR and to demonstrate that similar levels of FNR were present in cells harvested either during the exponential or stationary phase of growth. Lower levels of FNR were observed, however, when cultures were incubated for longer times (5 days or more) than the times listed above. The results shown in Table 1 (using purified preparations of FNR) show that the decrease in FNR activity observed in the NADPH:DCPIP oxidoreductase assay is also observed when the catalytic activity is measured using ferricyanide, ferredoxin:cytochrome *c*, or thio-NADP⁺ as electron acceptors. Thus the loss in specific activity of the de-activated form of the enzyme appears to be independent of the method of assay.

Since the loss of FNR activity during the stationary phase of cell growth is not due to protein degradation, other possible causes could be either: (1) the lack of some nutrient or the formation of an inhibitory metabolic product which would lead to enzyme inhibition or (2) the limitation of light reaching the cell, which is a consequence of the dense population of cells. An experiment was devised to test the second possibility by altering the light level illuminating a culture of cells in their exponential phase of growth and examining the effect on the level of FNR activity. The data in Fig. 1 show that FNR activity declines to a level ~ 20 % of its initial activity for a cell culture maintained in the absence of light, whereas the control culture kept continuously illuminated exhibited constant levels of enzyme activity. Photosynthetic particles prepared from the 'dark' culture at 100 h exhibited 50 % of the NADP⁺-photoreducing activity in the presence of added ferredoxin compared with the activity

Table 1. Comparison of catalytic activities and enzyme levels in FNR preparations isolated from cells in the exponential and in the stationary phases of growth

Catalytic assays were performed at 25 °C using published assay methods. The amount of FNR was determined spectroscopically, using ϵ_{458} 9.8 $\text{mm}^{-1} \cdot \text{cm}^{-1}$. Catalytic-centre activities were calculated from the estimated FAD content of the purified FNR preparations.

Growth phase	Catalytic-centre activity (min^{-1})					FNR (mg/g of cells)
	NADPH:Fe(CN) ₆ ³⁻ oxidoreductase	NADPH:DCPIP oxidoreductase	NADPH:ferredoxin:cytochrome <i>c</i> oxidoreductase	Trans-hydrogenase		
Exponential	30 700	6200	4400	2265		25
Stationary	12 500	2150	1800	1216		22

Fig. 2. Effect of light intensity on FNR activity in cultures of *A. variabilis*

Four aliquots of a culture of *A. variabilis* in the exponential phase of growth were exposed to different light intensities (*I*) and incubated for 7 days. After cells were harvested, crude cell extracts were prepared and FNR activity was assayed using the NADPH:DCPIP oxidoreductase assay. Activities were normalized to chlorophyll levels (units/mg of chlorophyll).

Fig. 3. Re-activation *in vivo* of FNR by culture dilution

A. variabilis cells were grown to late stationary phase to deactivate FNR activity. Cells were then diluted 5-fold in either fresh medium (●) or in medium obtained from stationary cell growth (○) and illuminated. FNR activity was determined by NADPH:DCPIP oxidoreductase assays on crude cell extracts obtained from aliquots taken at various times. A cell culture at the same density as stationary-phase cells served as control (▲). Specific activity is expressed in units/mg of total protein.

of particles isolated from cells maintained under continuous illumination (results not shown). In a separate experiment, cell cultures were incubated for 7 days under various light intensities. The results in Fig. 2 show that the level of FNR activity increased with increased levels of light. These experiments support the notion that the level of FNR activity is directly related to the intensity of light reaching the cell and suggest a possible mode of light regulation of FNR *in vivo*.

Re-activation of FNR *in vivo*

If the hypothesis of light regulation of FNR activity in *Anabaena* has any physiological validity, it should be possible to re-activate FNR activity by increasing the light intensity on cells containing appreciable levels of de-activated FNR. Such a re-activation can be demonstrated in two types of experiments. Illumination of cells that had lost FNR activity by incubation in the dark resulted in the restoration of FNR activity (results not shown). Cells that had been incubated in late stationary phase and had lost considerable levels of FNR activity were simply diluted and the resulting diluted cultures illuminated. The results in Fig. 3 show that the specific activity of FNR increases from 7- to 8-fold within a 30 h incubation period in cells diluted either into fresh medium or into medium obtained from a stationary-phase culture. A control culture of undiluted cells exhibited no

increase in FNR activity. The appearance of activity on dilution into re-utilized medium suggests that the existence of an endogenous inhibitor or a lack of an essential component is not the reason for the loss of FNR activity. Control experiments in which 50 µg of chloramphenicol (an inhibitor of protein synthesis in prokaryotes)/ml was added to the culture under the conditions described in Fig. 3 showed an identical pattern of FNR re-activation, an observation that demonstrates that the increase in FNR activity shown in Fig. 3 is not due to synthesis of new protein. This evidence was also supported by the immunological quantification of the FNR at the times indicated in Fig. 3 (data not shown). The reversible de-activation → re-activation of FNR in response to light intensity shown under '*in vivo*' conditions suggests that the enzyme is physiologically regulated in some unknown manner. Since it was possible to isolate reasonable quantities of the purified enzyme which appears to remain de-activated through the purification procedure used for the active form of the enzyme, a comparison of catalytic and chemical properties of the de-activated FNR with those of the active form was done to probe possible mechanisms of de-activation.

Molecular properties of purified de-activated FNR

Purified preparations of de-activated FNR were examined by a number of approaches to probe for any structural alterations that would account for the lowered activity. The molecular mass determined by gel-filtration chromatography on Sephadex G-100 was found to be identical with that of the active enzyme, namely 36 kDa, which indicates that no proteolytic degradation had taken place. In agreement with this conclusion is the finding that the *N*-terminal amino acid sequence for the de-activated FNR (22 residues) is identical with that found previously for the native FNR [12]. Analysis of the de-activated preparation for phosphorus content showed 2 mol of non-covalently bound phosphate/mol of enzyme (attributable to the bound FAD coenzyme) as found for the active form [12] and demonstrates that protein phosphorylation is not involved in the modulation of the catalytic activity. Isoelectric-focusing gel electrophoresis also demonstrated no major differences in pattern for both forms of the enzyme. Absorption spectral properties of the bound FAD (1 mol/mol of enzyme) were also identical with those of the native enzyme. The visible c.d. spectrum of the de-activated FNR (from 300 to 550 nm) showed the same shape as, but a somewhat lower intensity (15%) than, that exhibited by the active form (results not shown). Since the visible c.d. spectrum is a measure of the asymmetry of the bound flavin environment in the protein, these data suggest small alterations in the FAD-binding site on de-activation of FNR. Similar behaviour has been observed previously for the reversible de-activation of snake-venom L-amino acid oxidase [21]. A further alteration in the properties of the bound FAD in the de-activated form of the enzyme is the finding of about 16% neutral FAD semiquinone on reductive anaerobic titrations of the enzyme either by using dithionite as a reductant in the presence of low concentrations (1 µM) of Methyl Viologen and Benzyl Viologen to ensure that the semiquinone formed was a result of thermodynamic rather than kinetic stabilization. The native form of FNR has been found to form 5–6% neutral FAD semiquinone under the same conditions. These data also support the notion of an alteration of the binding environment of the bound FAD on FNR de-activation. The increased levels of neutral semiquinone observed could arise from alterations of the one-electron redox potentials of the bound FAD. Further work is required to support this possibility.

Table 2 summarizes results of comparative thiol titrations under various conditions for the active and de-activated forms of the enzyme. Under non-denaturing conditions, both preparations

Table 2. Reaction of Nbs₂ with the thiol groups of native and de-activated FNR

All reactions were performed in 10 mM-phosphate, pH 7.0, at 25 °C.

FNR sample	Thiol groups (mol/mol of protein)	<i>t</i> _{1/2} (min)
Native	0.03	—
Native + 8 M-urea	2.75	38
Reduced native + 8 M-urea	4.95	—
De-activated	0.5	—
De-activated + 8 M-urea	2.7	2
Reduced de-activated + 8 M-urea	4.95	—

Table 3. Comparison of *Anabaena* ferredoxin and flavodoxin binding affinities to native and de-activated FNR

Titration were performed in 50 mM-Tris/HCl, pH 8, at 25 °C.
Binding was measured by difference absorption spectra.

	<i>K_d</i> (μM)	
	Native FNR	De-activated FNR
Flavodoxin	8.5	18.6
Ferredoxin	3.6	64.2

of enzyme show less than one reactive thiol group with Nbs₂ as the modifying reagent, although the de-activated enzyme exhibited a higher level of thiol reactivity (0.5 group/mol of enzyme as against 0.03). The total thiol content of each enzyme preparation was identical on unfolding in 8 M-urea; however, the rate of reaction is markedly higher with de-activated FNR than with the active enzyme. These data suggest a greater degree of unfolding for the de-activated enzyme than for the active form. Both forms exhibited the same level of reactivity and stoichiometry of thiol modification after reduction of the single disulphide linkage with borohydride in 8 M-urea. One interpretation of these data is that de-activation is a result of a thiol ⇌ disulphide interchange such that the single disulphide bond in the enzyme occurs between different cysteine residues in the two forms of FNR. The active enzyme may contain a disulphide bond between residues remote from one another in the amino acid sequence such that appreciable structure still remains in the presence of 8 M-urea, which would account for the slower reactivity of the three free thiol groups. In the de-activated form the disulphide bond could occur between residues in closer proximity to one another, such

that the protein would unfold more extensively in urea and the increased accessibility of the three free thiol groups would account for their observed greater reactivity. Further support for this hypothesis requires differential labelling of the cysteine residues by radiolabelled reagents such as *N*-ethylmaleimide or iodoacetate and peptide purification and sequencing.

The above data suggest an alteration in FNR conformation on de-activation as a result of the putative rearrangement of the single disulphide cross-link which is sufficient to alter the environment of the FAD coenzyme in its binding site. Since FNR readily forms a complex with ferredoxin or with flavodoxin as its physiological electron donor, it was of interest to determine whether the de-activated form of the enzyme was still able to form such a complex. Ferredoxin and flavodoxin binding to FNR were monitored by alterations in the absorption spectral perturbations of the redox chromophores in the visible spectral region [12]. The data in Table 3 show that the deactivated form of FNR is still capable of binding ferredoxin or flavodoxin in a 1:1 stoichiometry; however, the binding affinities are quite different from those values for the active form. Ferredoxin is bound to de-activated FNR with a 16-fold weaker affinity than to the active form under the conditions of the experiment. The differences observed with flavodoxin are smaller, with a 2-fold decrease in binding for the deactivated form. Alterations of binding affinity of ferredoxin to the level observed could have physiological significance in the regulation of electron transport.

Steady-state kinetic properties of the deactivated enzyme

Although the results in Table 1 demonstrate a diminished catalytic activity for the de-activated form of the enzyme regardless of the method of assay, these values were obtained under 'standard' assay conditions at substrate concentrations known to be saturating only for the active form of FNR. The possibility remained that the de-activation process might involve an alteration in kinetic parameters, such as *K_m* values, for either the reducing or oxidizing substrates. Steady-state experiments were performed to measure the respective *K_m* and *k_{cat}* values for the catalytic properties of the de-activated enzyme with substrates in the standard assays used. The results of these measurements are shown in Table 4. By using ferricyanide or DCPIP as electron acceptors, no significant changes in *k_{cat}* are observed on comparison of the active with the deactivated forms of FNR. Significant alterations in the *K_m* values for NADPH are observed in which the de-activated form exhibits a 2–5 times higher value than the active form. By contrast, no alteration in *K_m* for NADPH is observed on comparison of activities in the ferredoxin-mediated reduction of cytochrome *c*. The major difference exhibited is the approx. 10-fold increase in *K_m* for ferredoxin for the de-activated form of the enzyme. This finding is consistent with binding studies (Table 3), where the binding affinity of

Table 4. Comparison of steady-state catalytic properties of the active (n-) and de-activated (d-) forms of *A. variabilis* FNR

Assay	Catalytic-centre activity (min ⁻¹)		<i>K_m</i> (μM)			
			NADPH		Acceptor	
	n-FNR	d-FNR	n-FNR	d-FNR	n-FNR	d-FNR
NADPH → ferricyanide	30 770	30 300	61	120	260	195
NADPH → DCPIP	6300	6200	5	28	—	—
NADPH → ferredoxin → cytochrome <i>c</i>	5560	5950	11.5	11.0	1.7	11.0
NADPH → thio-NADP ⁺	2270	1210	36	38	18	18

Fig. 4. Complex-formation between de-activated FNR and ferredoxin in 50 mM-Tris/HCl, pH 8, at 25 °C

To 850 μ l of 32 μ M de-activated FNR, increasing amounts of 0.39 mM-ferredoxin were added to a final concentration of 50 μ M. The Figure shows the experimental values (O) and the theoretical curve.

ferredoxin to the de-activated form is 16-fold lower than that of the active form. The only assay where a significant difference in k_{cat} is observed between the two forms of the enzyme is in the transhydrogenase assay (Table 4), where the K_m values for both electron donor and acceptor are unaffected by the level of enzyme activation. These results demonstrate that the decrease in activity observed on light limitation *in vivo* is expressed by increases in K_m values, which reflect alterations in conformation of the enzyme as shown from differences in thiol-reactivity (Table 2). The finding of differential effects on K_m (NADPH or ferredoxin) in the different assays reflects differences in mechanistic pathways and the relevant rate constants involved in expression of the K_m value. It is remarkable that Lineweaver-Burk plots of those reactions showed linear kinetics (results not shown), indicating that the electron-transfer reaction is catalysed by a low-activity FNR and not by a mixture of native and completely inactive enzyme. The experimental data used to calculate the binding constants (Table 3) are also consistent with the formation of one electrostatic complex between ferredoxin and FNR which contains one single binding site (Fig. 4). If native and completely inactive enzyme were present, the experimental data would indicate binding of ferredoxin to one enzyme with two binding sites with different affinities.

DISCUSSION

The regulation of a number of enzymes involved in photosynthetic pathways by light has been observed in species ranging from cyanobacteria to higher plants. Most of the enzymes known to be subject to light regulation are involved in reactions in the Calvin cycle, and the mechanism proposed in this regulation is via a thiol $\leftrightarrow$ disulphide interchange in which the ferredoxin/thioredoxin system is involved [22]. Photosynthetic enzymes involved in nitrate assimilation are also proposed to be regulated by a redox mechanism in which light intensity is involved [23].

FNR is a key enzyme in the distribution of Photosystem I reducing equivalents for either NADPH or ATP synthesis. Structural changes in FNR have been reported as a consequence of changes in the energy state of the thylakoid membrane or in the ionic composition of the stroma that occur during illumination [24]. Presumably such alterations could influence the conformational properties of the enzyme, resulting in the changes

in properties observed here for *Anabaena* FNR in response to alterations in light intensity. The results presented here agree with the observations reported by Nikolaeva & Osipova [9] for modulation of FNR activity in bean plants.

Comparison of the physical and catalytic properties of the active and de-activated forms of *Anabaena* FNR suggests no major alterations in structure; however, small alterations in the conformation of the enzyme take place which influence the binding affinity for ferredoxin, the K_m for ferredoxin and the K_m for NADPH, depending on the assay used. The thiol titration data suggest this alteration could be due to a thiol $\leftrightarrow$ disulphide interchange that takes place in an intramolecular manner. Further confirmatory evidence for this suggestion must await specific labelling of the thiol groups in the two forms of the enzyme and placing them in the amino acid sequence. The events leading to this postulated mechanism in response to alterations in light intensity to the cell still await further investigation. We have observed re-activation of the de-activated form of FNR *in vitro* in response to freezing and thawing of dilute solutions of the enzyme. The level of re-activation achieved is dependent on the concentration of enzyme and does not occur if such treatment is carried out in the presence of glycerol (20%, v/v). Interpretation of such experiments are difficult; however, observations such as this would be consistent with an alteration in protein conformation on freezing, which could facilitate an intramolecular thiol $\leftrightarrow$ disulphide interchange, leading to a form of the enzyme exhibiting K_m values of the active form. Incubation of the de-activated form of FNR with dithiothreitol did not lead to any observable re-activation, although a number of concentrations and conditions were tested.

The properties described here for the de-activated form of FNR are similar to those described by Carrillo & Vallejos [7] for the de-activated spinach enzyme generated during light $\leftrightarrow$ dark transitions. Both systems exhibit lower affinities for reductive and oxidizing substrates. Differential behaviour between the spinach and cyanobacterial enzymes are observed with respect to reactivity with thiol reagents. The similarities observed, however, suggest that future work directed towards elucidating the molecular basis for FNR regulation in *Anabaena* may be relevant to our understanding of FNR regulation in higher plants.

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