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Lysine residues on ferredoxin-NADP$^+$ reductase from *Anabaena* sp. PCC 7119 involved in substrate binding

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Ferredoxin-NADP$^+$ reductase from *Anabaena* sp. PCC 7119 is chemically modified by pyridoxal 5'-phosphate. The incorporation of 2±0.3 mol pyridoxal 5'-phosphate/mol ferredoxin-NADP$^+$ reductase inhibited NADPH-cytochrome *c* reductase activity by up to 95% while 55% of diaphorase activity still remained. Considerable protection against inactivation was afforded by ferredoxin. Chymotryptic cleavage of the modified enzyme was performed, the peptides were separated by high performance liquid chromatography, and the peptides containing pyridoxamine 5'-phosphate were identified by their fluorescence and by their absorbance at 325 nm. Three major labelled peptides were found. Their sequences were comprised of residues 46–54, 231–235 and 289–295. Lys-53 and -294 were the residues which presented the highest degree of modification and seem to be involved in the ferredoxin binding site of ferredoxin-NADP$^+$ reductase from *Anabaena* sp. PCC 7119.

Ferredoxin-NADP$^+$ reductase; Chemical modification; Essential lysine residue

1. INTRODUCTION

Ferredoxin-NADP$^+$ reductase (FNR;EC 1.18.1.2) is the terminal electron-carrier of the photosynthetic electron transport-chain. The enzyme from *Anabaena* sp. PCC 7119 has been recently crystallized and the crystals shown to diffract up to 1.9 Å [1]. Work on its structure by X-ray diffraction is in progress while its amino acid sequence has already been published [2]. Recently, the three-dimensional structure of the spinach FNR has been reported [3] as the domains of interaction with FAD and the 2'-phospho-AMP (a non-physiological substrate of the enzyme). The importance and characteristics of complex formation of this protein with its substrates (NADP$^+$ and ferredoxin, Fd) has been recently reviewed [4].

Chemical modification studies have shown the presence of essential arginine residues located in the NADP$^+$ and Fd binding sites of the *Anabaena* FNR [5]. Apart from this, several lysine residues have also been reported to be involved in substrate binding [3,6–9] in the spinach enzyme. Since the sequence homology between *Anabaena* sp. PCC 7119 and spinach does not go beyond 50% identity and, at the same time, most of the lysine residues which have been reported to be involved in substrate binding in the spinach FNR are replaced by arginines in the *Anabaena* sp. PCC 7119 enzyme, it would be interesting to know whether there are other essential lysine residues in this FNR which might be responsible for substrate binding.

Since pyridoxal 5'-phosphate (PLP) is capable of forming a Schiff base with the ε-amino groups of lysyl residues in proteins as well as with the α-amino function of amino terminal residues, and this adduct (PLP–amino acid) is easily detected by spectroscopic methods, we have chosen this reagent to modify FNR. In the present paper we describe the effects of PLP on *Anabaena* sp. PCC 7119 FNR activities and the identification of the residues which were the target of the modification.

The results we present here, taken together with data concerning the three-dimensional structure of the protein, will allow the determination of the structural requirements of the domain of contact of proteins involved in electron transfer reactions through protein–protein interaction. At the same time, they give clear hints as to those amino acids which could be the target of site-directed mutagenesis.

2. MATERIALS AND METHODS

2.1. *Materials*

Anabaena sp. PCC 7119 FNR, Fd and flavodoxin were purified to homogeneity as previously described [10]. Their concentrations were determined spectrophotometrically using an extinction coefficient of 9.4 $mM^{-1} \cdot cm^{-1}$ at 459 nm for FNR, 7.2 $mM^{-1} \cdot cm^{-1}$ at 423 nm for Fd [10] and 9.4 $mM^{-1} \cdot cm^{-1}$ at 464 nm for flavodoxin [11]. PLP was purchased from Sigma. All other chemicals were commercially available and of reagent grade.

Abbreviations: FNR, ferredoxin-NADP$^+$ reductase; Fd, ferredoxin; PLP, pyridoxal 5'-phosphate; EDC, 1-ethyl(3-(3-dimethylaminopropyl)carbodiimide; DCPIP, dichlorophenolindophenol; TFA, trifluoroacetic acid; PCC, Pasteur Culture Collection.

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2.2. *Enzymatic assays*

The diaphorase (EC 1.8.1.4) activity of the FNR was assayed with DCPIP as electron acceptor [12]. The FNR-dependent NADPH cytochrome *c* reductase (EC 1.6.99.3) activity was assayed as described [13]. Both activities were performed in a thermostated KONTRON Uvikon 860 spectrophotometer at 23°C to assay FNR.

2.3. *Modification of lysine residues*

FNR (4.8 μM) was incubated in the dark in a reaction mixture containing 10 mM potassium phosphate, pH 8.0, 5% glycerol, 0.1 mM EDTA and 20 mM PLP at 25°C. Aliquots were removed at various time intervals and checked for residual enzymatic activity. When stabilization of the Schiff base was required, after 20 min incubation, PLP was neutralized by the addition of a freshly prepared solution of $NaBH_4$ to give a final concentration of 10 mM, and then the reaction mixture was incubated for an additional 15 min. After removal of excess reagents on a small sephadex G-25 column, the stoichiometry of the enzyme–PLP adduct was determined by measuring the increase in absorbance at 325 nm. A molar absorptivity of 9720 $M^{-1} \cdot cm^{-1}$ at 325 nm [14] was used to calculate the amount of PLP bound to the enzyme.

2.4. *Other assays*

Binding of modified FNR to Fd and $NADP^+$ was monitored using absorption difference spectroscopy as described by Foust et al. [15] using a thermostated KONTRON Uvikon 860 spectrophotometer. Covalent complex formation of FNR with Fd or flavodoxin was followed by SDS-PAGE after incubation of equimolar amounts of the proteins in the presence of EDC (5 mM) for 4 h [16]. Proteins were stained with 0.1% Coomassie blue R-250 in 30% methanol and 10% acetic acid in distilled water, and destained in the same solution without the dye. Isoelectrofocusing was assayed in homogeneous polyacrylamide gels covering the pH range 4–6.5, in a Pharmacia Fast system. Proteins were stained with 0.02% Coomassie blue R-250 in 30% methanol and 10% acetic acid in distilled water and 0.1% (w/v) $CuSO_4$.

2.5. *Peptide analysis*

Chymotryptic digests were carried out at 37°C overnight with 3:100 (w/w) protease/FNR in 0.2 M *N*-methylmorpholine/acetate buffer, pH 8.2, 2.5 M guanidinium chloride. The digest was freeze-dried, lyophilized and finally re-dissolved in 0.1% aqueous trifluoroacetic acid (TFA). Chymotryptic peptides were resolved by HPLC. The chromatograph consisted of two Waters M6000A pumps, a Waters 680 automated gradient controller, Waters 990 photodiode array detector with a dynamic range from ultraviolet to the visible (UV–VIS) region (200–500 nm) and a Waters 420 fluorescence detector (λ_{ex} 338 nm, λ_{em} 400 nm) based on a NEC APC III personal computer. Sample injections were performed with a Waters U6K universal injector. Reversed-phase HPCL was performed with an Aquapore C_8 RP-300 7μ column (250 × 7.0 mm i.d., from Brown Lee). The column was eluted with acetonitrile gradients containing 0.1% TFA and operated at room temperature at a flow-rate of 0.7 ml/min. Peptides were sequenced in a Beckman Sequencer (model 890D). The PTH-amino acids were identified and quantified on a RP-HPLC system based upon C_{18} column (Nova Pak) and gradient elution with 3.5 mM sodium acetate:acetonitrile (16:3), adjusted to pH 5 as buffer A and isopropanol:water (3:2) as buffer B.

3. RESULTS AND DISCUSSION

Incubation of FNR from *Anabaena* sp. PCC 7119 with PLP results in the inactivation of the enzyme as measured by monitoring enzyme activity (Table 1). The NADPH diaphorase and NADPH–cytochrome *c* reductase (Fd-dependent) activities were checked in order to monitor the modification. Both activities decreased rapidly in such a way that after 1 min there was practically no further change in the activity. For this reason the kinetic behaviour of the process was not fully explored. These results suggest that lysine residues and/or the α-amino terminal residue may be important for enzyme activity. The observation that NADPH–cytochrome *c* reductase activity was considerably more affected than diaphorase activity (where binding of Fd is not required), suggests that some lysine residues which are involved in the binding of FNR to the Fd have been modified. Dialysis or dilution of the incubation mixture produce the active enzyme again. When samples incubated with PLP were reduced with $NaBH_4$ and the excess or reagents removed, a further decrease in NADPH–cytochrome *c* reductase activity by about 20% was detected, while diaphorase activity further decreased by only 5%. The $NaBH_4$ incubation of the native enzyme did not produce any observable changes in its activity. These observations suggest that PLP forms a Schiff base with an ε-amino group which is stabilized upon treatment with $NaBH_4$. $NaBH_4$ prevents the hydrolysis of the imine bond in the PLP–enzyme complex upon dilution to give active enzyme in the assay mixture [17]. Protection experiments (Table 1) showed that Fd afforded protection against inactivation in both activities, while $NADP^+$ had only a small protective effect, indicating that the region modified must be involved in the contact with Fd.

The number of residues modified by the PLP treatment followed by $NaBH_4$ reduction was estimated spectrophotometrically as described in Materials and Methods and found to be 2±0.3 per mol of FNR. Fluorescence spectrum of the PLP-modified enzyme produced an emission maximum at 390 nm when the excitation wavelength was 298. This is indicative of the binding of the fluorescent PLP molecule to the enzyme.

Table 1

Effect of PLP incubation on FNR activities

Sample	% of initial activity	
	Diaphorase	NADPH–cytochrome *c* reductase
After addition of PLP		
FNR	60	25
FNR + $NADP^+$	75	30
FNR + FD	95	75
After $NaBH_4$ reduction		
FNR	55	4
FNR + $NADP^+$	70	9
FNR + FD	90	65

Inactivation experiments were performed as described in Materials and Methods. Where indicated, $NADP^+$ or Fd were 17 mM and 240 μM, respectively. The 100% activity corresponded to the assay with native FNR. Time of incubation 200 min. A control of native FNR, previously incubated with $NaBH_4$, presented 98% activity.

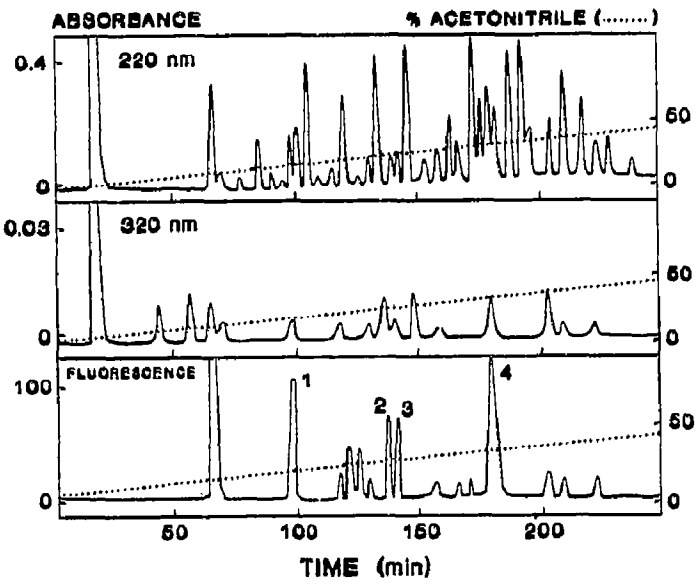

Fig. 1. High-performance liquid chromatography separation of the chymotryptic peptides from PLP-FNR. After modification of FNR, 20 nmol of the sample were dialyzed against 0.2 M *N*-methylmorpholine/acetate buffer, pH 8.2, digested with chymotrypsin and the resulting peptides were chromatographed. The peptides were eluted with a 255-min linear gradient from 0–45% acetonitrile. Elution was at a flow-rate of 0.7 ml/min at room temperature. The peptide map was monitored by absorbance at 220 and 320 nm and by fluorescence at 400 nm (with excitation at 338 nm).

In order to check if the modification of FNR with the lysil-binding reagent, PLP, produced any changes in the ability of the enzyme to interact with its substrate, Fd, two different tests were used: (i) the ability to form the non-covalent complex, which is regarded as an intermediate of the catalytic cycle; and (ii) the covalent linkage of FNR to Fd (or flavodoxin) upon treatment with the carbodiimide EDC which has been reported [16] to occur with the native enzyme. It was observed that incubation of FNR with PLP followed by $NaBH_4$ reduction almost completely prevented the formation of the non-covalent complex with Fd as determined by differential spectrophotometry measurements (not shown), while the non-covalent complex formation with $NADP^+$ was just slightly disturbed. The modified enzyme was also shown to be unable to form a covalent complex with both Fd and flavodoxin, so it can be deduced that the site of interaction of FNR with the electron carrier protein is blocked by the PLP molecules. Isoelectric focusing of native and treated FNR was also carried out. The same four bands which are observed in the native enzyme, indicative of a not yet well-established microheterogeneity, were found, but at slightly lower isoelectric points (range 4.3–4.78 as compared to the range 4.6–5.1 for the native protein). All these data are indicative of the loss of positive charges (lysine residues) during incubation of FNR with PLP. Since it is believed that positively charged amino acid residues on FNR are responsible for the electrostatic interactions with its substrates, it is likely that those 2 or 3 out of the 24 Lys residues present in the enzyme which have become selectively modified are essential residues for the interaction of the FNR with its substrates.

The HPLC chymotryptic peptide map of the modified protein is shown in Fig. 1. Since the PLP-ε-*N*-Lysine adduct absorbs at 320 nm and presents a high fluorescence, both methods have been employed to detect which peptides have incorporated this chemical. One major fluorescent peak was observed in the chymotryptic map (4 in Fig. 1), which does not appear in the native enzyme chromatogram. Among the other peaks which exhibit both fluorescence and absorbance at 320 nm only three more were selected for analysis, due to the fact that the others appeared in the native enzyme or because the amount was small enough to be considered the result of an unspecific modification reaction or cleavage.

Peaks 1–4 (Fig. 1) were sequenced. Fig. 2 shows the sequences found and their alignment with other FNRs. Peaks 1 and 2 corresponded to the same lysil residue modified but with a different cleavage of the protein. Peak 1 corresponded to residues 286–295 in the *Anabaena* sp. PCC 7119 sequence where there are three lysine residues which are fully conserved in all the species compared. Lys-294 was, nevertheless, the only one which had incorporated PLP. This position corresponds to the COOH-terminal region of the protein and is located in the NADPH domain. Peak 3 corresponded to residues 221–235 in *Anabaena* sp. PCC 7119 enzyme, and Lys-227 was the target of the modification. This peptide contains two lysine residues in the higher plant enzyme sequences, but neither of them corresponds to Lys-227 in *Anabaena* sp. PCC 7119 FNR in the alignment. In the spinach enzyme, chemical modification experiments have shown Lys-244 to be involved in NADPH binding [18] and the three-dimensional model reported by Karplus [3] shows that it is directly involved in its binding. In *Anabaena* sp. PCC 7119 FNR this function could be carried out by Arg-233, since an arginine residue which binds NADPH in this enzyme has been reported [5], and/or also by Lys- 227. The loop which contains these residues in the spinach enzyme is highly exposed to the solvent and has been shown to move during ligand binding [3]. It can therefore be concluded that Lys-227 in *Anabaena* sp. PCC 7119 FNR is modified by treatment with PLP but it seems to be involved in the binding to the substrate NADPH rather than to Fd. The peptide which displayed the highest degree of fluorescence (4 in Fig. 1) corresponded to residues 46–54 in the *Anabaena* sp. PCC 7119 FNR sequence and the residue modified was Lys-53. This residue corresponds to Arg-49 in *Spirulina* FNR and an arginine residue is only two positions ahead in all the other FNR sequences compared (Arg-71 in spinach, Arg-67 in *Mesembryanthemum crystallinum* and Arg-65

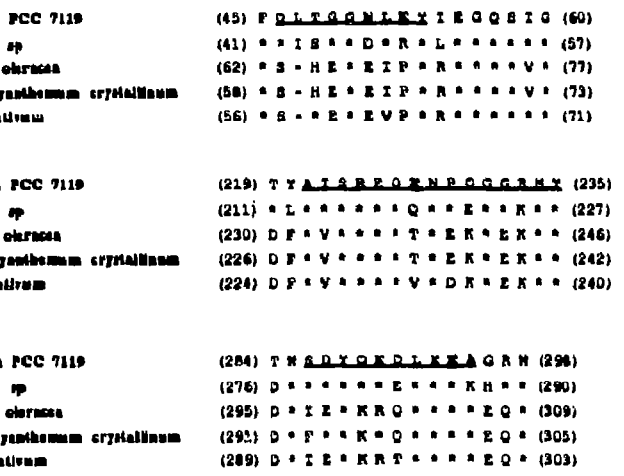

Fig. 2. Assignment of the modified residues in the *Anabaena* sp. PCC 7119 FNR sequence. Alignment of regions of the amino acid sequence of FNR from *Anabaena* sp. PCC 7119 [2], *Spirulina* sp. [21], Spinach [22], *Pisum sativum* [23] and *Mesembryanthemum crystallinum* [24]. Asterisks indicate exact matches of the sequences regarding *Anabaena* sp. PCC 7119. Hyphens are packing characters introduced to align the sequences. The residues in bold type are those which have been modified. The sequenced peptides are underlined.

in *Pisum sativum*, Fig. 2). When this region is filled in the three-dimensional structure obtained for the spinach enzyme [3] it is located in the FAD binding domain. It corresponds to a loop whose last side chain residue, Ser-75 (Ser-59 in *Anabaena* sp. PCC 7119 FNR), forms the surface surrounding the edge of the dimethyl-benzyl ring of FAD, which is the only part of the flavin molecule exposed to the solvent [19]. Cross-linking studies have implicated Lys-85 and/or Lys-88 in the spinach enzyme (which are conserved in all the FNRs and correspond to Lys-69 and Lys-72 in the *Anabaena* sp. PCC 7119 FNR) in the binding to Fd [20]. Although we have not found any modification of these residues in *Anabaena* FNR, it does not necessarily mean that they are not involved in the binding with Fd. They could have a diminished reactivity toward PLP as a consequence of their chemical environment.

Since more drastic effects have been found after treatment of *Anabaena* sp. PCC 7119 FNR with PLP in those reactions where interaction with Fd was assayed, we propose that Lys-53 and Lys-294 are involved in the binding to Fd. The data presented in this paper would place the Fd binding domain of the *Anabaena* sp. PCC 7119 FNR in the cleft between the two domains of the protein. This has also been suggested by computer graphic studies of the interaction between *Spirulina* Fd and spinach FNR [3], as was previously predicted by chemical modification studies in the spinach enzyme [7,8,18].

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