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Flavin photochemistry in the analysis of electron transfer reactions: role of charged and hydrophobic residues at the carboxyl terminus of ferredoxin–NADP$^+$ reductase in the interaction with its substrates

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Abstract

The enzyme Ferredoxin-NADP$^+$ reductase participates in the reductive side of the photosynthetic chain transferring electrons from reduced Ferredoxin (Fd) (or Flavodoxin (Fld)) to NADP$^+$, a process that yields NADPH that can be used in many biosynthetic dark reactions. The involvement of specific amino acids in the interaction between the two proteins has been studied using site-directed mutagenesis. In the present study, the participation of charged (H299), polar (T302) or hydrophobic (V300) amino acid residues that are in the NADP$^+$-binding domain of the reductase have been examined by analyzing its C-terminal region, which is located close to the active site. Stopped-flow and laser flash photolysis results of the reaction in which these mutant proteins participate show very little differences with respect to the wild-type protein. These results suggest that the NADPH-binding domain of the reductase has little effect on the processes of recognition and electron transfer to (and from) Fd or Fld, according to the recently reported crystallographic structure of the FNR/Fd complex. © 2002 Published by Elsevier Science B.V.

Keywords: Electron transfer; Ferredoxin; NADP$^+$

1. Introduction

During the light phase of photosynthesis, two electrons are withdrawn from a water molecule and are used to reduce NADP$^+$ to NADPH [1]. The three proteins that participate in this electron transfer (ET) chain in the cyanobacterium *Anabaena* have been the subjects of numerous studies. These are ferredoxin (Fd), an 11-kDa iron–sulphur protein, the FAD-containing enzyme ferredoxin–NADP$^+$ reductase (FNR), and the 20-kDa FMN-containing flavodoxin (Fld) that replaces Fd upon limitation of iron in the culture medium [2]. The reaction between Fd and FNR is consistent with the (minimal) two-step mechanism:

$$Fd_{rd} + FNR_{ox} \overset{K_d}{\rightleftharpoons} [Fd_{rd}\text{- - -}FNR_{ox}] \rightarrow Fd_{ox}^{k_{et}} + FNR_{rd}$$

in which K_d and k_{et} represent the dissociation constant for the intermediate complex and the ET rate constant, respectively. Several charged and hydrophobic residues on the surfaces of Fd and FNR have been proposed to be required for the formation of the ET complex [3–6]. Thus far, all FNR residues that have been shown to present marked effect on the interaction with the ET protein were located in the so-called flavin domain (residues 1–139). In the present study, we have determined if charged or hydrophobic residues located at the NADP$^+$-binding domain of the reductase were also involved in the Fd/FNR interaction by analysing its C-terminal region, which is located close to the active site.

2. Experimental

The H299A, H299K, H299F, V300F, and T302V FNR mutants were produced and purified as previously described [6,7]. NADPH-dependent diaphorase activity was assayed with DCPIP. Stopped-flow experiments were performed under anaerobic conditions using an Applied Photophysics SX17.MV spectrophotometer interfaced with an Acorn 5000 computer using the SX.18MV software of Applied Photophysics as previously described [7]. Samples of reduced FNR were prepared by photoreduction of the

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Table 1
Kinetic parameters of the reaction between FNR and its substrates

FNR	Diaphorase activity			Stopped-flow[a] $FNR_{rd}+Fld_{ox}$	Laser flash photolysis[b] $Fd_{rd}+FNR_{ox}$	
	k_{cat} (s^{-1})	K_m^{NADPH} (μM)	k_{cat}/K_m (s^{-1} μM^{-1})	k_{obs} (s^{-1})	K_d (μM)	k_{et}[c] (s^{-1})
WT	81.5 ± 3.0	6.0 ± 0.6	13.5 ± 0.5	2.5 ± 1.0 1.0 ± 1.0	9.3 ± 0.7[d]	6200 ± 400
H299A	118 ± 1.3	7.15 ± 0.6	16.7 ± 1.7	0.8 ± 0.1 0.15 ± 0.02	17.9 ± 1.5	9700 ± 800
H299K	121 ± 10	5.4 ± 0.7	22.5 ± 0.5	0.74 ± 0.05 0.14 ± 0.03	17.9 ± 1.5	9700 ± 800
H299F	99 ± 2.8	6.0 ± 0.47	16.5 ± 1.3	2.27 ± 1.2 0.44 ± 0.12	9.3 ± 0.7	6200 ± 400
V300F	92.8 ± 2.0	4.38 ± 0.31	21.2 ± 1.5	7.7 ± 0.7 1.08 ± 0.09	9.3 ± 0.7	6200 ± 400
T302V	128 ± 3.9	6.4 ± 0.5	20.0 ± 1.6	0.9 ± 0.08 0.18 ± 0.03	17.9 ± 1.5	9700 ± 800

[a] The samples were mixed in the stopped flow spectrometer at a concentration ratio 1:1, 10 μM final concentration for each protein.
[b] FNR was titrated into solutions containing 30 μM Fd. Solutions also contained 1 μM 5-dRf and 1 mM EDTA in 4 mM potassium phosphate buffer (pH 7.0). The ionic strength was adjusted using aliquots of 5 M NaCl.
[c] Second order rate constants for the Fd/FNR interaction were estimated from the initial slopes of k_{obs} vs. [FNR] curves for WT, H299F, and T302V. Values for H299A and H299K were calculated from the linear plots.
[d] Taken from Ref. [11].

protein-bound redox center in 50 mM Tris/HCl, pH 8.0, also containing 20 μM EDTA and 2–4 μM dRf. Reactions were performed at 13 °C. The observed rate constants (k_{obs}) were calculated by fitting the data to a biexponential equation. The laser flash photolysis system has been described previously [8] as has the photochemical system [9] used to initiate ET. Samples were deoxygenated by bubbling for 1 h with O_2-free Ar prior to laser photolysis. The saturation kinetics obtained in the FNR concentration dependencies of the observed rate constant (k_{obs}) allowed the kinetic constants, k_{et} (ET rate constant) and K_d (dissociation constant for the transient Fd_{rd}/FNR_{ox} complex), to be obtained by fitting the kinetic data to the exact solution to the differential equation describing the (minimal) two-step mechanism [10].

3. Results and discussion

All the FNR mutants were obtained at similar levels to those of the WT. No major differences were found in the UV–visible and CD spectra indicating that no major structural changes had been introduced by the mutations. The kinetic parameters observed for the different FNR mutants in the diaphorase assay (Table 1), K_m and k_{et}, indicate that none of the mutations introduced at H299, V300, and T302 produces any remarkable effect on the processes of interaction and ET from NADPH to FNR. Analysis of the reduction of Fld by reduced FNR using stopped-flow kinetic techniques also indicates that the ability to transfer electrons from reduced FNR to the ET protein is only affected

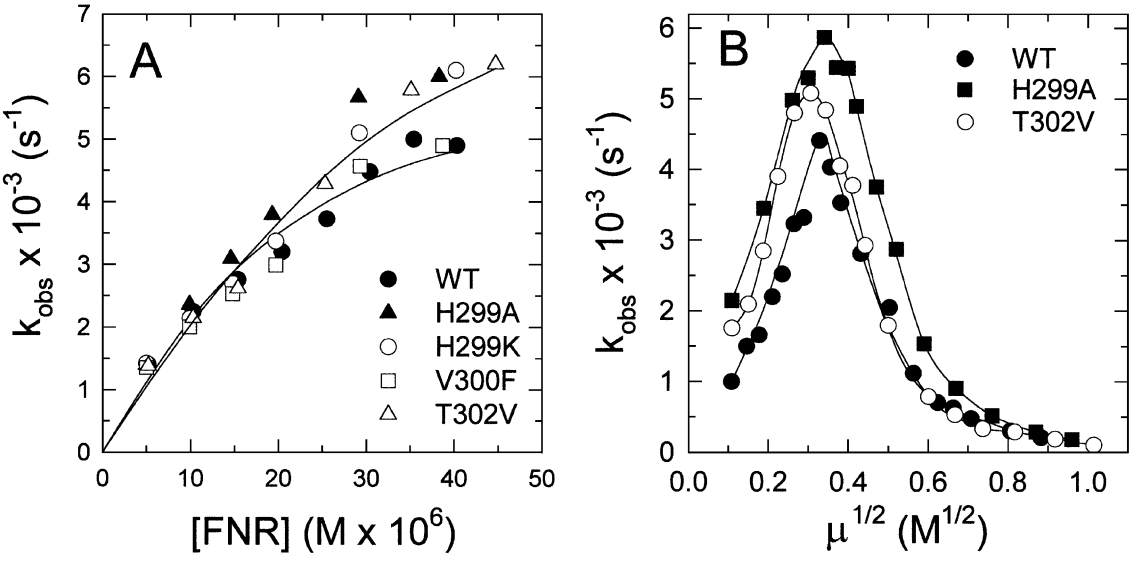

Fig. 1. (A) FNR concentration dependencies of the k_{obs} for the reduction of WT and mutant FNR species by reduced Fd at 100-mM ionic strength. (B) Ionic strength dependencies for the reduction of WT and mutant FNR forms by reduced Fd.

partially since only minor changes are determined in the k_{obs} of the processes as compared to those reported for the WT (Table 1). The ET interactions between Fd_{rd} and the oxidised forms of the FNR mutants were very similar to those involving WT FNR, as determined by laser flash photolysis/time-resolved absorbance measurements. The FNR concentration dependencies of k_{obs} are shown in Fig. 1A for the reduction of H299A, H299K, V300F, T302V, and WT FNR by Fd_{rd} at $\mu = 100$ mM. The results for H299F (not shown) and V300F (Fig. 1A) were essentially the same as those obtained for WT, and therefore, the kinetic constants K_d and k_{et} were taken to be identical to the values determined for WT FNR (Table 1). The other mutants behaved very similarly to each other (Fig. 1A) and had a k_{et} value that was slightly larger than that obtained for WT FNR and a K_d for the intermediate complexes two-fold larger than the WT value (Table 1). The ionic strength dependencies of k_{obs} for all these mutants are similar to those of WT FNR. The only slight difference is the peak k_{obs} values for H299A and T302V, which were approximately 30% and 15% larger than the peak WT value, respectively (Fig. 1B). Although these differences are not large, this indicates that these mutations may result in transient complexes in which the mutual orientation of the proteins is somewhat more conducive to ET than for WT FNR. In fact, the replacement of T302 by V must prevent the formation of an H-bond interaction at the FNR/Fd interface, which has been shown to stabilise the *Anabaena* crystallographic complex [12].

In this work, three amino acids residues, the positively charged H299, the polar T302, and the hydrophobic V300, that belong to the carboxyl end and are located in the NADPH-binding site of FNR, were replaced by others having different properties. The stationary and prestationary kinetic parameters determined for all these mutants indicate that they undergo the oxidation/reduction reaction in a similar manner as the WT. These results can be taken as an indication that these amino acid residues are not involved to a large extent in either the interaction between FNR and the ET protein (Fd or Fld), or in the ET process. On the contrary, it has been previously shown that residues located at the same distance from FAD but located within the FAD-binding domain, Lys75, Leu76, and Leu78, are important for proper orientation and ET between FNR and the carrier protein [2,4,5]. Therefore, the data presented in this work confirm that the NADPH-binding domain has little effect on processes of recognition and ET to Fd and Fld, as suggested from the recently reported crystallographic structure of the FNR/Fd complex [12].

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