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Flavodoxin: A compromise between efficiency and versatility in the electron transfer from Photosystem I to Ferredoxin-NADP$^+$ reductase

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ABSTRACT

Under iron-deficient conditions Flavodoxin (Fld) replaces Ferredoxin in *Anabaena* as electron carrier from Photosystem I (PSI) to Ferredoxin-NADP$^+$ reductase (FNR). Several residues modulate the Fld interaction with FNR and PSI, but no one appears as specifically critical for efficient electron transfer (ET). Fld shows a strong dipole moment, with its negative end directed towards the flavin ring. The role of this dipole moment in the processes of interaction and ET with positively charged surfaces exhibited by PSI and FNR has been analysed by introducing single and multiple charge reversal mutations on the Fld surface. Our data confirm that in this system interactions do not rely on a precise complementary surface of the reacting molecules. In fact, they indicate that the initial orientation driven by the alignment of dipole moment of the Fld molecule with that of the partner contributes to the formation of a bunch of alternative binding modes competent for the efficient ET reaction. Additionally, the fact that Fld uses different interaction surfaces to dock to PSI and to FNR is confirmed.

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1. Introduction

Ferredoxin NADP$^+$ reductase (FNR) catalyses the electron transfer (ET) from Photosystem I (PSI) to NADP$^+$. In plants, electrons are transferred from PSI to FNR by Ferredoxin (Fd), but in most cyanobacteria and some algae, under iron deprivation, Flavodoxin (Fld) can substitute for Fd. Fd and Fld are different in size, sequence, folding and cofactors ([2Fe–2S] for Fd and FMN for Fld). However, both can function in the midpoint potential range ∼−400 mV [1] and, alignment on the basis of their electrostatic potentials indicates overlapping of their strong negative potentials around their redox centres [2]. The surfaces of PSI and FNR where Fd and Fld bind contain mainly positive patches (Fig. SP1), suggesting that electrostatic forces will contribute to the complex formation step preceding ET [3–5].

In cyanobacteria PSI assembles as a trimer, each monomer containing 12 proteins and more than 100 cofactors [6]. Electrons flow from the P700 reaction centre through a series of carriers to reach the terminal [4Fe–4S] clusters, F_X, F_A and F_B. The PsaC, PsaD and PsaE subunits contribute to the positively charged solvent accessible stromal site of PSI (Fig. SP1A). The PsaC subunit binds F_A and F_B and cannot be deleted without loss of PSI activity; PsaD is important for electrostatic steering of Fd and Fld; and PsaE has been implicated in controlling lifetime and stabilisation of the PSI:Fd complex, in cyclic ET and/or in a ternary complex with FNR [5,7]. K35 from PsaC on the *Chlamydomonas reinhardtii* PSI [8], as well as K106 from PsaD [9–11] and R39 on PsaE in *Synechocystis* [12], play key roles in binding of the protein acceptor. The nature of several *Anabaena* Fld side chains has been shown to contribute to the formation of a transient PSI:Fld complex. However, replacements had minor effects on the ET process itself, and for some mutants mechanisms involving no-transient complex formation were as efficient as the WT one [4,13–15]. In *Anabaena* FNR, the surface around the FAD group presents patches of positively charged residues (Fig. SP1B). R16, K72, R264 and especially K75 are required as positively charged, while L76 and L78 must be hydrophobic, for the efficient interaction with both Fd and Fld [1,16–18]. Key counterpart residues to those were found on the Fd surface, namely F65 and E94 [16,17]. However, individual replacements of residues on the Fld surface indicated that they were not involved in crucial specific interactions with FNR [18–20].

Therefore, the interaction of Fld with its partners appears to be less specific than that of Fd. This is also suggested by docking models showing that Fld could orientate in different ways on the FNR surface without significantly altering the distance between the methyl groups of FAD and FMN and, keeping the molecular dipoles on FNR and Fld nearly collinear [21]. The parameters that regulate the Fld movement between its docking site on PSI and that on FNR during Fld-dependent

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photosynthetic ET are further studied in this work. Residues, T12, E16, E20, E61, E67, D126 and D144 have been replaced with positively charged or neutral side chains. The cooperative action of these residues has been analysed in the E16K/E61K, E16K/D126K, E16K/E20K/D126K and E16K/E61K/D126K/D150K Flds. Finally, K2 and K3, residues producing one of the few accumulations of positive potential on the Fld surface, were simultaneously replaced by producing the K2A/K3A and K2E/K3E Flds. The effect of these mutations on the association and ET processes of Fld with PSI and FNR are discussed on the bases of the changes produced in the surface electrostatic potential and the dipole moment magnitude and orientation, as well as on the structural bases that regulate midpoint-reduction parameters.

2. Materials and methods

2.1. Site directed mutagenesis, protein expression and purification

Mutations at K2, K3, T12, E16, E20, E61, D126 and D150 were introduced into the pTrc99a plasmid encoding the *Anabaena* Fld gene by PCR-based site-directed mutagenesis using the Stratagene QuikChange Kit (see Supplementary material, SP) [14]. Multiple mutations were sequentially introduced after several rounds of mutagenesis. Mutations were verified by DNA sequence analysis and MALDI-TOF mass spectrometry. WT and mutated pTrc99a-Fld plasmids were overexpressed in *Escherichia coli* TG1 strain. Recombinant *Anabaena* PCC 7119 WT FNR, WT Fld and mutated Flds were purified from LB cultures of IPTG-induced *E. coli* [14]. UV/Vis spectra and SDS-PAGE were used as purity criteria. *Anabaena* PCC 7119 PSI particles were obtained as described [22]. The P700 content in PSI samples was calculated from the photoinduced absorbance changes at 820 nm, ε=6.5 mM^{-1} cm^{-1}. Chlorophyll concentration was determined according to Arnon [23]. The chlorophyll/P700 ratio of the PSI preparation was 104/1. The same batches of PSI and proteins were used throughout this study.

2.2. Spectral analyses

UV–visible spectral measurements were carried out on a Kontron Uvikon 942, an Agilent HP 8453 or a Varian Cary 100 Bio spectrophotometer. Measurements were carried out at 25 °C in 50 mM Tris/HCl, pH 8.0. Extinction coefficients of Fld$_{ox}$s were determined as described [24]. Other extinction coefficients were; $\varepsilon_{FNRox,458\ nm}$=9.4 mM^{-1} cm^{-1}, $\varepsilon_{Cytcox,550\ nm}$=8.4 mM^{-1} cm^{-1} and $\varepsilon_{Cytcrd,550\ nm}$=29.5 mM^{-1} cm^{-1}. Stepwise anaerobic photoreduction of Flds was carried out in the presence of 2 µM 5-deazariboflavin (5-dRf) and 3 mM EDTA [13] and used to determine the percentage of maximum semiquinone stabilised and its extinction coefficient (see SP). Dissociation constants (K_d), extinction coefficient changes ($\Delta\varepsilon$) and free energy ($\Delta G°$) for complex formation between WT FNR$_{ox}$ and the Fld$_{ox}$ variants were obtained by difference absorption spectroscopy [14]. Fitting of the experimental data to the theoretical equation for a 1:1 complex allowed the calculation of K_d and $\Delta\varepsilon$. Errors in the determined K_d and $\Delta\varepsilon$ values were ± 15%, and ± 10% for $\Delta G°$. The FNR NADPH-cytochrome *c* reductase activity was assayed by using the Fld mutants as electron carrier from FNR to cytochrome *c* (Cytc) (Sigma) [25]. Errors in the estimated K_m and k_{cat} values were ± 15% and ± 10%, respectively.

2.3. Oxido-reduction potential determinations

Midpoint reduction potentials were determined in a protein solution combining potentiometric measurements of the oxido-reduction potential with optical measurements [13,15]. Experiments were carried out by photoreduction of ~40 µM Fld in the presence of 2 µM 5-dRf, 3 mM EDTA and ~2 µM of each mediator dye at 25 °C in 50 mM Tris/HCl pH 8.0, in an anaerobic cuvette, using a gold electrode and a saturated calomel electrode (details in SP). Errors in the $E_{ox/sq}$ and $E_{sq/hq}$ were estimated to be ±5 mV.

2.4. Fast kinetic stopped-flow measurements

Stopped-flow measurements were carried out under anaerobic conditions using an Applied Photophysics SX17.MV spectrophotometer [14]. Reduced samples of FNR and Fld were prepared by photoreduction. Reactions were followed after mixing the proteins at a ~1:1 molar ratio with a final concentration of ~10 µM for each protein, in 50 mM Tris/HCl pH 8.0 at 10 °C. Processes between FNR and Fld were followed at 600 nm and, those between Fld$_{hq}$ and Cytc$_{ox}$ at 550 nm. The apparent observed rate constants (k_{ap}) were calculated by fitting the data to mono- or bi-exponential processes. Each kinetic trace was the average of 20–30 measurements. Each experiment was repeated three times. Maximal errors in the determined k_{ap} values were ±15%.

2.5. Fast kinetic laser-flash absorption spectroscopy measurements

ET processes between PSI and Fld were followed at 580 nm by laser-flash absorption spectroscopy [14]. The standard reaction mixture contained in 1 ml, 20 mM Tricine/KOH, pH 7.5, 0.03% β-dodecyl maltoside, an amount of PSI-enriched particles equivalent to 50 µg of chlorophyll ml^{-1}, 0.05 µM phenazine methosulfate, 2 mM $MgCl_2$, 2 mM sodium ascorbate, and Fld at the indicated concentration. The experiments at increasing $MgCl_2$ concentrations were carried out with a final Fld concentration of 20 µM. Each kinetic trace was the average of 40–50 measurements, with 30 s intervals between flashes. Observed rate constants (k_{obs}) were obtained from 2 to 3 different experiments and analyses were carried out according to the two-step reaction mechanism [14]. Error in the determination was <20%.

2.6. Crystal growth, data collection, and structure refinement

Crystals of K2A/K3A, E16K/E61K and E16K/E61K/D126K/D150K Flds were obtained by the hanging drop method: for K2A/K3A and E16K/E61K Fld, 1.5–2 µl of a 5 mg/ml protein solution in Tris/HCl pH 8.0 were mixed with 1 µl of reservoir solution consisting of 32% PEG 4000, 0.2–0.3 M $MgCl_2$ and 0.1 M Tris/HCl pH 8.5; for E16K/E61K/D126K/D150K Fld, 1 µl of a 5 mg/ml protein solution buffered with 0.1 M Tris/HCl pH 8.0 was mixed with 1 µl of reservoir solution consisting of 22% PEG 8000 and 0.2 M $CaCl_2$ buffered with 0.1 M MES, pH 6.5. The X-ray data set of the K2A/K3A Fld crystal was collected at the BM16 beamline of the ESRF on an ADSC Quantum210 detector with a wavelength of 0.97918 Å. Data for E16K/E61K and E16K/E61K/D126K/D150K Flds were collected with a Kappa 2000 CCD detector using a graphite monochromated CuK$_\alpha$ radiation generated by a Bruker–Nonius rotating anode. Data collection and refinement statistics are shown in Table SP1. The V_m are 1.95, 1.99 and 1.96 Å^3/Da and over 36.8%, 38.2% and 37.4% solvent contents for K2A/K3A, E16K/E61K and E16K/E61K/D126K/D150K Flds, respectively. Data were processed, scaled and reduced with HKL2000 [26]. The mutant structures were solved by molecular replacement, using the program MOLREP [27] and the native Fld$_{ox}$ structure (PDB code 1flv). Refinement was made using the programs CNS [28] and REFMAC [29] and manual model building by the graphics program "O" [30]. In E16K/E61K Fld tight non-crystallographic restraints between the four different protein chains of the asymmetric unit were applied during refinement, which were gradually loosened in the final rounds. Stereochemistry of the models was checked using PROCHECK [31]. Atomic coordinates are deposited at the PDB: 3esz for K2A/K3A Fld, 3esy for E16K/E61K Fld and 3esx for E16K/E61K/D126K/D150K Fld.

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