Insights into the design of a hybrid system between *Anabaena* ferredoxin-NADP⁺ reductase and bovine adrenodoxin

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The opportunity to design enzymatic systems is becoming more feasible due to detailed knowledge of the structure of many proteins. As a first step, investigations have aimed to redesign already existing systems, so that they can perform a function different from the one for which they were synthesized. We have investigated the interaction of electron transfer proteins from different systems in order to check the possibility of heterologous reconstitution among members of different chains. Here, it is shown that ferredoxin-NADP⁺ reductase from Anabaena and adrenodoxin from bovine adrenal glands are able to form optimal complexes for thermodynamically favoured electron transfer reactions. Thus, electron transfer from ferredoxin-NADP⁺ reductase to adrenodoxin seems to proceed through the formation of at least two different complexes, whereas electron transfer from adrenodoxin to ferredoxin-NADP⁺ reductase does not take place due because it is a thermodynamically

Many biological processes depend on protein–protein electron transfer (ET) reactions, where the specific interaction of a reduced protein with its oxidized counterpart is required [1,2]. The fact that many of the proteins involved in these reactions are able to interact with different partners raises the question about the nature of their interaction surfaces. This can be demonstrated by proteins like ferredoxins (Fd), small [2Fe–2S] proteins that are involved in a multitude of reactions in microorganisms, plants and animals. In the case

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Fax: + 34 9/0/02123, 1ei.: + 34 9/0/01288, E-mail: gomezm@posta.unizar.es. http://wwwbioq.unizar.es/ *Abbreviations*: FNR, ferredoxin-NADP⁺ reductase; FNR_{ox}, FNR in the oxidized state; FNR_{rd}, FNR in the reduced state; FNR_{sq}, FNR in the semiquinone state; Fd, ferredoxin; Fd_{ox}, Fd in the oxidized state; Fd_{rd}, Fd in the reduced state; dRf, 5-deazariboflavin; WT, wild-type; *E*, midpoint reduction potential; ET, electron transfer; Adx, adrenodoxin; Adx_{rd}, adrenodoxin in the reduced state; Adx_{ox}, adrenodoxin in the oxidized state; Adx(4–108), truncated adrenodoxin comprising residues 4–108; AdR, adrenodoxin reductase; CYP11A1, cytochrome P450scc. nonfavoured process. Moreover, by using a truncated adrenodoxin form (with decreased reduction potential as compared with the wild-type) ferredoxin-NADP⁺ reductase is reduced. Finally, these reactions have also been studied using several ferredoxin-NADP⁺ reductase mutants at positions crucial for interaction with its physiological partner, ferredoxin. The effects observed in their reactions with adrenodoxin do not correlate with those reported for their reactions with ferredoxin. In summary, our data indicate that although electron transfer can be achieved in this hybrid system, the electron transfer processes observed are much slower than within the physiological partners, pointing to a low specificity in the interaction surfaces of the proteins in the hybrid complexes.

Keywords: adrenodoxin; electron transfer; ferredoxin-NADP⁺ reductase; protein–protein interaction.

of Anabaena, a photosynthetic nitrogen-fixing cvanobacterium, Fd is involved in the recognition of the photosystem I and also of several enzymes such as ferredoxin-NADP⁺ reductase (FNR), nitrate and nitrite reductase, glutamate synthase or thioredoxin reductase [3]. This suggests that although the overall structures of these proteins differ widely, their Fd interaction surface should contain some common features. Moreover, it is known that in Anabaena, FNR can recognize not only Fd but also flavodoxin (Fld), a small FMN-containing protein that is synthesized under conditons of iron deficiency when it replaces Fd in the ET from photosystem I to FNR [4]. The fact that these two proteins, with different structures, sizes and redox cofactors, can be recognized by FNR using the same binding site also supports the idea of the similarity in the recognition mechanisms for ET proteins [5]. Additional examples can also be found in the superfamily of the cytochromes P450. In the mitochondrial steroid hydroxylating cytochrome P450 systems these enzymes catalyse the hydroxylation of a range of substrates by receiving electrons from small electron transport chains. Starting from NADPH the reduction equivalents are transferred via an FAD containing reductase (AdR) to the one-electron carrier adrenodoxin (Adx), which supplies electrons to the different P450s [6]. An example of such a P450 is CYP11A1, which converts cholesterol to pregnenolone, the precursor of all steroid hormones.

Moreover, as a first step in the design of novel enzymatic systems, recent investigations are aimed to redesign already

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existing systems. Therefore, it is feasible to consider using proteins to work in ET chains for which they were not naturally synthesized. In the present study we have tried to increase the knowledge of the parameters that keep running the ET reactions in proteins by the combination of two biological ET chains involved in the production of biological compounds of important economic value: the photosynthetic electron transport chain involved in NADPH production and the cytochrome P450 chain that catalyses steroid hormones synthesis in adrenal glands. Thus, we have examined these requirements for productive complex formation and ET, by using a heterologous system that consists of cyanobacterial FNR and adrenal bovine Adx. Anabaena PCC 7119 FNR contains a noncovalently bound FAD group and its main physiological function is the transfer of two electrons from two molecules of reduced Fd to NADP⁺ [7]. FNR site-directed mutants have been studied providing a large amount of information about its interaction and ET properties to Fd, Fld and NADP⁺ [8–11]. Three-dimensional structures of Anabaena wild-type (WT) FNR, several of its mutants and of its complexes with both NADP⁺ and Fd have been reported [8,11-15]. A basic, K75, and two hydrophobic residues, L76 and L78, have been shown to be crucial for the formation of a functional complex with the partner protein [8,13]. Bovine Adx, a [2Fe-2S] vertebrate-type Fd, is a key component of the steroid hormone-producing system in the adrenal mitochondria. Three-dimensional structures for the WT and a truncated Adx (4-108) [16,17] have been reported, the first one suggesting the presence of functional dimers. Although sequence identity between plant- and vertebrate-type Fd is less than 23% [18], comparison of their structures has revealed that the N terminus of Adx is structurally similar to that of Anabaena Fd (see Fig. 2 in [19]). Moreover, in both Fd-type proteins the residues involved in the interaction with their reductases are located at similar positions on the molecular surface and are coupled to the iron centre via structurally similar hydrogen bonds. However, despite these similarities, it is interesting to point out the different arrangement of the [2Fe-2S] centres of these Fds. The cyanobacterial Fd presents an increased shielding from the solvent of the active Fe in ET when compared with that of Adx. Such different cluster environments must contribute to the lowered reduction potential exhibited by the cyanobacterial Fd (-384 mV for Anabaena Fd vs. -273 mV for adrenal Adx) [10,18,20,21]. Finally, in both systems, it is assumed that the clearly asymmetric charge distribution at the surfaces of the reductase and the Fd-type electron carrier would produce a strong long-range electrostatic attraction that appears to be a determinant for the initial approach. However, any further tight binding required for efficient ET will be governed by nonpolar interactions [5,13,18,22].

Materials and methods

Biological material

WT, K75E, L76S, L78S, L78D, L78F, L78V and V136S FNR were prepared as described previously [8,10,13]. WT Adx, Adx(4–108) and CYP11A1 were produced following standard protocols [23]. All measurements reported were performed in 50 mM Tris/HCl pH 8.0.

Analysis of the interaction between Adx_{ox} and FNR_{ox} by differential absorption spectroscopy

Dissociation constants (K_{ds}) of the complexes between FNR_{ox} and either Adx_{ox} or Adx(4–108)_{ox} were obtained as described previously [10]. These experiments were performed in tandem cuvettes containing 20 μ M FNR_{ox} into which aliquots of 1 mM Adx_{ox} were added stepwise.

Steady-state kinetic measurements

Reactions between the different FNR_{rd} forms and Adx_{ox} were followed by steady-state methods using a HP8452 single beam photodiode array spectrometer. Reactions were carried out under anaerobic conditions at 13 °C in a twocompartment anaerobic cell, thereby allowing the two proteins to be stored separately while degassing and to be reduced independently. Samples were made anaerobic by successive evacuation and flushing with O2-free Ar. FNR was fully reduced by adding a 25 molar excess of NADPH under positive pressure of Ar. A constant FNR concentration of 8 µM and different Adx concentrations, in the range $8-160 \mu M$, were used. After recording a baseline with the preincubated NADPH/FNR mixture, present in the cellmeasuring compartment, the reaction was initiated by mixing the contents of the two compartments, and followed over 1200–1800 s by recording the visible spectra every 15 s. Absorbance changes at 414 nm were chosen to determine rate constants, as at this wavelength maximal changes of the amplitudes were observed. The desired ionic strength for salt titration experiments was adjusted by the addition of aliquots of a 5-M NaCl stock solution, buffered in Tris/HCl 50 mм pH 8.0.

Reduction of CYP11A1 by the hybrid FNR/Adx system was checked using the same methodology. In this case, 8 µM FNR and 8 µM Adx were initially mixed in the cell measuring compartment and 3 µM CYP11A1 was placed in the second compartment. After the samples were made anaerobic, an excess of NADPH was added to the FNR/Adx mixture to allow Adx reduction via the NADPH prereduced FNR. Simultaneously, CO-gas was bubbled into the cell through a capillary syringe (for 20 min) to reach CO-saturation. After recording a baseline with the NADPH/FNR/Adx mixture, the contents of the two compartments were mixed in order to initiate the reduction of CYP11A1 by Adx. Time resolved spectra were then recorded to follow the appearance of the typical absorption spectrum of the CO-ferrous CYP11A1 complexed form, characterized by absorbance decreases at 390, 430 and 480 nm and by the appearance of a peak at 450 nm which exhibits a large extinction coefficient [24].

Reduction of the different FNR species by Adx, either WT or Adx(4–108), was also checked under steady-state conditions following the methodology described above. In this case, reduced Adx was prepared by photoreduction via the highly reductive dRfH[•] radical generated by light irradiation of the sample also containing dRf (1–2 μ M) and EDTA (2 mM) [25]. Final FNR concentration was always 8 μ M. Different [Adx_{ox}]/[FNR_{rd}] ratios were used. The baseline was collected with photoreduced Adx prior to mixing the contents of the compartments. Time dependent spectra between 400 and 600 nm were then recorded in order to follow the Adx reoxidation by FNR.

Stopped-flow kinetic measurements

Stopped-flow measurements were carried out under anaerobic conditions using an Applied Photophysics SX17.MV spectrophotometer interfaced with an Acorn 5000 computer. Data were analysed using the sx.18 MV software of Applied Photophysics as described previously [10,26]. Samples were made anaerobic before being introduced into the stopped-flow syringes. FNR species were reduced by preincubation with an excess of NADPH under anaerobic conditions. Reduced Adx forms were prepared by photoreduction as described above. Between five and 10 independent measurements were collected and averaged for each reaction. Reactions were followed at both 414 nm and 600 nm, where Adx reoxidation/reduction and FNR semiquinone formation can be followed, respectively. A constant final FNR concentration of 8 µM was used. [Adx]/[FNR] ratios are indicated elsewhere for each experiment.

The observed rate constants (k_{obs}) were calculated by fitting the data to mono- or bi-exponential equations. Initial rate constants (V_0) were also determined from the slope of the linear region at the beginning of every reaction trace. Standard deviation for both values is $\pm 10\%$.

Results

Interaction between Adxox and FNRox

Spectral perturbations appear upon formation of 1 : 1 complexes of FNR with ET proteins such as Fd, Fld and rubredoxin [27]. *In vitro* studies also revealed that Adx forms 1 : 1 complexes with both AdR and CYP11A1 [28–30]. In the present study, spectral changes were observed by differential absorption spectroscopy upon mixing of FNR_{ox} with either Adx_{ox} or Adx(4–108)_{ox} (data not shown). Such changes were dependent on Adx_{ox} concentration and fit to the theoretical equation for a 1 : 1 interaction, allowing the determination of a K_d value of 25 ± 3 µM for the [FNR_{ox}:Adx_{ox}] complex and of 17 ± 2 µM for the [FNR_{ox}:Adx(4–108)_{ox}] complex (Table 1).

Study of the kinetics of reduction of WT Adx by FNR_{rd}

Stopped-flow kinetic studies indicate that reduction of Adx_{ox} by FNR_{rd}, as followed by the kinetic transients at 414 nm (Fig. 1A), was taking place over a period of time of

at least 1000 s and therefore can be analysed under steadystate conditions. Moreover, upon analysing the reaction at shorter time scales an absorbance increase was observed within 10 s of mixing (Fig. 1A, inset), which might be due to a reorganization of the initial complex prior to ET itself. Steady-state conditions were used to analyse the reaction further, and the spectral changes shown in Fig. 1B were observed with time. The maximum absorption values at 414 nm and 450 nm, both characteristic of Adxox, observed in the first spectrum recorded after the reaction is initiated (Fig. 1B, top line) indicate that Adx reduction by FNR_{rd} , does not take place within the experimental dead time. However, over a period of more than 10 min a significant decrease in absorbance is observed at both wavelengths, consistent with Adx_{ox} (E = -273 mV) [18] reduction either by FNR_{rd} (E = -312 mV) or by the subsequent FNR_{sq} (E = -338 mV) generated [31], as both are thermodynamically favoured processes.

Figure 2A shows the kinetic transients observed at 414 nm, corresponding to Adx reduction by FNR_{rd}, at $[Adx_{ox}]/[FNR_{rd}]$ ratios ranging between 1 : 1 and 20 : 1. The observed amplitudes, at each protein ratio, are consistent with the extinction coefficient changes expected for the transition from oxidized to reduced Adx. Traces obtained upon mixing of equimolar amounts of FNR_{rd} and Adx_{ox} fit to a monoexponential process with a k_{obs} value of 0.003 s⁻¹ (Fig. 2B). However, addition of increasing amounts of Adx, while keeping the FNR concentration constant, resulted in kinetic traces that are better described by a bi-exponential fit (Fig. 2C,D). Moreover, the k_{obs1} and k_{obs2} values obtained diminish upon increasing the Adx_{ox} concentration (Fig. 3A). This observation is not consistent with a minimal two-step mechanism involving complex formation prior to the ET reaction. In this case an increase in the k_{obs} value would be expected with increasing Adxox concentration, finally leading to saturating conditions that would be associated with an asymptotic curve. With regard to the total amplitude of both processes, A_1 and A_2 , which represent the extent to which the reaction is taking place, we observe a clear increase of the amplitude with increasing Adx_{ox} concentration (Fig. 3B). However, whereas a much larger proportion of the total Adx seems to be reduced following the slower process at [Adxox]/[FNRrd] ratios up to 7:1 (A_2 larger than A_1), both amplitudes become nearly identical at higher [Adxox]/[FNRrd] ratios

Table 1. Thermodynamic and kinetic parameters for the FNR/Adx interaction. ND, not determined; NR, no reaction observed.

Reductase/				
protein carrier system	<i>K</i> _d ^a (µм)	Reaction	$k_{\rm obs}~({\rm s}^{-1})$	
WT FNR/Adx	25	$FNR_{rd} + Adx_{ox}$	0.003^{d}	
		$Adx_{rd} + FNR_{ox}$	NR	
WT FNR/Adx(4–108)	17	$FNR_{rd} + Adx(4-108)_{ox}$	ND	
		$Adx(4-108)_{rd} + FNR_{ox}^{e}$	$k_{\rm obs1} \ 0.013$	
			$k_{\rm obs2} \ 0.002$	
WT FNR/Fd ^b	4	$FNR_{rd} + Fd_{ox}$	> 700	
		$Fd_{rd} + FNR_{ox}$	6200	
AdR/Adx ^c	0.77	$AdR_{red} + Adx_{ox}$	7.2	

^a Standard deviation for all shown K_d values is $\pm 15\%$. ^b Data from [10]. ^c Data from [34]. ^d Data at ratio 1 : 1. ^e Data at [Adx_{rd}(4–108)]/[FNR_{ox}] ratio 4 : 1.



Fig. 1. Time-course and spectral changes for the anaerobic reaction between FNR_{rd} and Adx_{ox} as followed by stopped-flow and under steady-state conditions. (A) Time course followed by stopped-flow at 414 nm. [Adx_{ox}]/[FNR_{rd}] ratio 3 : 1. Final concentration of FNR was 10 μ M. The inset shows the first seconds of the reaction. (B) Spectral changes observed in the 400–650 nm range when followed under steady-state conditions. [Adx_{ox}]/[FNR_{rd}] ratio 3 : 1. Final concentration of FNR was 8 μ M. The spectrum on the top corresponds to the first one recorded after mixing. Both reactions were carried out at 13 °C in 50 mM Tris/HCl pH 8.0.

(Fig. 3B). To sum up, the plot of the initial rate constants (V_0), which represents the initial rate of the reaction, vs. the Adx concentration shows an almost linear correlation (Fig. 3A), suggesting that the formation of the optimal complex between the two proteins limits the ET process.

When analysing the effect of ionic strength on the interaction between FNR_{rd} and Adx_{ox} (Fig. 3C), it was found that k_{obs1} and V_0 showed a subtle biphasic ionic



Fig. 2. Time-course for the anaerobic reaction between FNR_{rd} and Adx_{ox} using a constant FNR concentration and increasing $[Adx_{ox}]/[FNR_{rd}]$ ratios. (A) Time-course for the anaerobic reaction between FNR_{rd} and Adx_{ox} as followed under steady-state conditions at 414 nm and $[Adx_{ox}]/[FNR_{rd}]$ ratios: 1 : 1 (\bigcirc), 2 : 1 (\square), 3 : 1 (\blacktriangle), 4 : 1 (\times), 7 : 1 (\bigcirc), 10 : 1 (\diamondsuit), 15 : 1 (\blacklozenge), 20 : 1 (+). Final concentration of FNR was 8 μ M. Residuals for the fitting of (B) the $[Adx_{ox}]/[FNR_{rd}] = 1 : 1$ trace to a single exponential, (C) the $[Adx_{ox}]/[FNR_{rd}] = 20 : 1$ trace to a bi-exponential. Reactions were carried out at 13 °C in 50 mM Tris/HCl pH 8.0.

strength dependency with maximal values around 20 mM ionic strength (0.14 $M^{1/2}$), whereas k_{obs2} was almost independent. Such slight biphasic dependence might be ascribed to the formation of an initial electrostatically bound complex which needs subsequent reorganization to adapt a more favourable orientation for efficient ET. Such behaviour has also been described for other systems including Fd_{rd}/FNR_{ox} [22,26,32]. Thus, the decrease in k_{obs1} and V_0 observed above 40 mm ionic strength $(0.2 \text{ m}^{1/2})$ might be attributed to the disruption of the electrostatic interactions between the oppositely charged proteins, by reducing the long-range electrostatic forces responsible for the initial approach of the proteins. However, the increase of either k_{obs1} or V_0 observed up to 20 mm $(0.14 \text{ M}^{1/2})$ is only small. This suggests that either the longrange electrostatic interactions, which account for the initial protein-protein encounter, are rather weak, or that after breaking of the long-range interactions, short-range specific interactions at the protein-protein interface are not strong



Fig. 3. Kinetic parameters for the anaerobic reaction between FNR_{rd} and Adx_{ox}. Data calculated from steady-state spectra recorded in Fig. 2A at 414 nm. Adx concentration dependence of (A) k_{obs1} , k_{obs2} and V_0 (lines are drawn in for clarity only, they do not represent fittings) and (B) the corresponding amplitudes; $A_1 (\blacksquare)$ and $A_2 (\triangle)$. (C) Ionic strength dependence of k_{obs1} , k_{obs2} and V_0 for a 3 : 1 [Adx_{ox}]/ [FNR_{rd}] ratio. The ionic strength was adjusted using aliquots of 5 M NaCl. Final concentration of FNR was 8 μ M. k_{obs1} (\Box), k_{obs2} (\triangle) and V_0 (\bullet).

enough to provide a correct orientation between the redox centres for efficient ET.

Reduction of CYP11A1 by the hybrid NADPH/FNR/Adx ET chain

After interaction and productive reduction of Adx_{ox} by FNR_{rd} had been shown, it was of interest to study the



Fig. 4. Spectral changes observed for the formation of the CO-CYP11A1_{rd} complex upon CYP11A1 reduction by the NADPH/FNR/ Adx system. The inset shows the time course of the CYP11A1 reduction followed at 450 nm. CO saturated solutions contained 8 μ M FNR, 200 μ M NADPH, 8 μ M Adx and 3 μ M CYP11A1. Reactions were carried out at 13 °C in 50 mM Tris/HCl pH 8.0.

ability of the FNR/Adx ET system to efficiently reduce a cytochrome P450 enzyme, for example CYP11A1. The transfer of the first electron to the CYP11A1 by the oneelectron carrier Adx can be followed spectroscopically. In the reduced state cytochrome P450 binds CO yielding a complex that shows a typical absorbance band at 450 nm [33]. Time-sequential spectra recorded after addition of CYP11A1 to an anaerobic CO-saturated sample containing the reaction mixture FNR_{rd}/Adxox gave rise to a peak at 450 nm together with absorbance decreases at 390, 430 and 480 nm (Fig. 4). These spectra can be explained by the formation of such a CO-CYP11A1 ferrous complex [33]. Thus, we generated an artificial but functional ET chain composed of Anabaena FNR, bovine Adx and bovine CYP11A1. The time course of the reaction followed at 450 nm fit to a mono-exponential process with a $k_{\rm obs}$ 0.031 s⁻¹ and a V_0 of 0.0012 s⁻¹ for the FNR dependent ET from Adx to CYP11A1 under the experimental conditions used (Fig. 4, inset).

Reduction of FNR by Adx_{rd}

When examining the reverse reaction between photoreduced WT Adx and FNR_{ox} under anaerobic conditions, no absorbance changes, even at periods as long as 1200 s, attributable to a modification in the oxidation state of any of the redox centres were detected (Fig. 5A). All the recorded spectra showed the characteristic peak of FNR_{ox} centred at 458 nm, indicating that ET from Adx_{rd} to FNR_{ox} does not take place. This result was not unexpected as the reduction potentials reported for both proteins indicate a low thermodynamic probability of ET from Adx_{rd} to FNR_{ox} [18,21,31].



Fig. 5. Spectral changes observed in the 400–600 nm spectral range for the anaerobic reaction between (A) FNR_{ox} and WT Adx_{rd} and (B) FNR_{ox} and $Adx(4-108)_{rd}$. The reactions were followed under steadystate conditions over a period of 1200 s. $[Adx_{rd}]/[FNR_{ox}]$ ratio 4 : 1. The lower spectrum corresponds to the first one recorded after mixing. The inset in (B) shows the time-course dependence for the absorbance at 414 nm. For both reactions final FNR concentrations were 8 μ M and were carried out at 13 °C in 50 mM Tris/HCl pH 8.0.

Reduction of FNR by Adx(4-108)_{rd}

A truncated mutant of Adx, Adx(4–108), prepared by deleting residues 1–3 and 109–128, has been shown to possess a much more negative reduction potential than WT Adx (–344 mV vs. –273 mV) [18,21]. Taking into account the two independent one-electron reduction potential values for FNR, $E_{ox/sq} = -338$ mV and $E_{sq/rd} = -312$ mV [31], reduction of FNR_{ox} to any of both states, semiquinone or reduced, by Adx(4–108)_{rd} would be thermodynamically favoured, which might lead to a redox reaction after complex formation. Therefore, spectral changes were analysed after mixing the truncated Adx(4–108)_{rd} with FNR_{ox}. The spectra obtained (Fig. 5B) are consistent with reoxida-

tion of Adx(4–108)_{rd} by FNR_{ox}. The time course of the reaction (Fig. 5B, inset), followed at 414 nm and using a 4 : 1 [Adx_{rd}(4–108)]/[FNR_{ox}] ratio, best fit to a bi-exponential process with k_{obs} of 0.013 s⁻¹ and 0.002 s⁻¹ (Table 1) and V_0 of 0.0007 s⁻¹.

Moreover, the time resolved steady-state spectra showed an absorption band in the 600 nm region that remained almost constant during the steady-state measurement. Such an absorption band is consistent with the presence of FNR_{sq} and it is already present at the very beginning of the reaction, indicating that its formation takes place within the dead time of the steady-state experiment. Stopped-flow experiments were then performed to further investigate the formation of semiguinone. As expected, reduction of FNR by Adx(4-108)_{rd} produces an increase in absorbance at 600 nm during the first seconds after mixing (Fig. 6A). As this wavelength is an isosbestic point for Adx_{ox}/Adx_{rd}, the changes observed can be attributed only to the conversion of FNRox to FNRsq. The observed amplitudes increased with rising Adx concentration, implicating that productive [Adx(4–108)_{rd}:FNR_{ox}] complex formation is proportionally



Fig. 6. Time-course and kinetic data for the anaerobic reaction between FNR_{ox} and $Adx(4-108)_{rd}$ as followed by stopped-flow. (A) Transients obtained at 600 nm and at $[Adx_{ox}]/[FNR_{rd}]$ ratios: 1 : 1 (\triangle), 3 : 1 (\blacklozenge), 5 : 1 (\square), 7 : 1 (\blacksquare). (B) Adx concentration dependence of the k_{obs} (\bullet) and V_0 (\square) values calculated from transients at 600 nm (lines are only drawn in for clarity, they do not represent fittings). Final concentration of FNR was 8 μ M. Reactions were carried out at 13 °C in 50 mM Tris/HCl, pH 8.0.

FNR form	$k_{\rm obs}$ (s ⁻¹) for the mixing of FNR _{rd} with		$k_{obs} (s^{-1})$ for the mixing of FNR _{ox} with				
	Adx _{ox} ^b	Fd _{ox} ^c	Adx _{rd} ^b	$\mathrm{Fd_{rd}}^{c}$	$E_{\rm ox/rd}^{a}$ (mV)	$E_{\rm ox/sq}~({\rm mV})$	$E_{\rm sq/rd}~(\rm mV)$
WT	0.003	d	NR	d	-325	-338 ^a	-312 ^a
K75E	0.01	< 0.001	NR	2.7 0.5	-305	-312 ^a	-298 ^a
L76S	0.01	NR	0.014	NR	-305	-318 ^f	-282^{f}
L78S	0.005	0.41	0.012	d	-286	-299^{f}	-273^{f}
L78D	0.01 ^e	0.03	0.01	15.2	-302	-315 ^f	-289^{f}
L78F	0.02	240	0.01	146	-307	-320 ^f	-294^{f}
L78V	0.035	> 600	NR	d	ND	ND	ND
V136S	0.03	1	NR	160 d 100	-305	-318	-292

Table 2. Steady-state kinetic parameters for the interaction of several FNR forms with Adx. Data for the FNR/Fd systems as well as reduction potential values for the FNR mutants are shown for comparison. ND, not determined; NR, no reaction observed.

^a Data from [13,31]. ^b k_{obs} values determined from steady-state kinetic experiments at 414 nm at an [Adx]/[FNR] ratio of 1 : 1. ^c Data from [8,13]. ^d Reaction occurred within the instrumental death time. ^e A lag phase is observed at 414 nm until 200 s; the k_{obs} was estimated after this phase. ^f Data estimated from the $E_{ox/rd}$ value and the percentage of maximal semiquinone stabilized [31].

enhanced with higher Adx(4-108)rd concentration (Fig. 6A). Transients at 600 nm best fit to a monoexponential process with k_{obs} values slightly decreasing with increasing $Adx(4-108)_{rd}$, whereas V_0 values indicate slightly initial faster processes under such conditions (Fig. 6B). Taking into account the above observations: (a) there is a continuous reoxidation of Adx(4-108)rd during the reaction, and (b) the amount of FNR_{sq} formed remains constant, a mechanism in which FNR_{ox} is sequentially reduced through the semiquinone state by two independent $Adx(4-108)_{rd}$ molecules can be proposed. Thus, the first ET process would account for the fast increase in absorbance at 600 nm, corresponding to FNR_{sq} formation, observed by stoppedflow, while the slower process would correspond to reduction of the FNR_{sq} to the hydroquinone state by a second Adx(4–108)_{rd}. This mechanism would suggest that upon consumption of FNRsq by the second process, the same amount of FNR_{sq} is produced by the first one. This is consistent with the high K_d values proposed for the interaction of FNR and Adx and with the low amount of semiquinone stabilized by FNR taking into account its $E_{\text{ox/sq}}$ and $E_{\text{sq/rd}}$ values [31].

Reaction of different FNR mutants with Adx

Reactions of K75E, L76S, L78S, L78D, L78F, L78V and V136S FNR forms with Adx have also been investigated. Stopped-flow kinetic studies indicated that the reaction of any of these FNR_{rd} forms with Adx_{ox} is slow enough to be analysed under steady-state conditions (data not shown). A significant decrease in absorbance at 414 and 450 nm (data not shown) was observed for the reaction of all these FNR_{rd} mutants with Adx_{ox} , consistent with Adx_{ox} reduction by ET from FNR_{rd}, and only slight alterations in the k_{obs} values for the process were observed with regard to the WT FNR reaction (Table 2). Thus, whereas ET from FNR_{rd} to Adx seems to be slightly enhanced when using K75E, L76S, L78F, L78V or V136S FNRs, L78S behaves similarly to WT FNR. The kinetic observed for the reaction with L78D

FNR (data not shown), is noticeable. For this reaction a lag phase with no absorbance changes (200 s) is observed before the reaction is initiated, indicating that the accumulation of an obligatory intermediate takes place prior to ET.

When analysing the reverse reaction (i.e. reduction of FNR_{ox} by Adx_{rd}) no absorbance changes were detected for the reactions with K75E, L78V and V136S FNRs (data not shown), as for that with WT FNR (Fig. 5A), indicating that ET from Adx_{rd} to any of these FNR_{ox} forms does not take place. However, mixing of L76S, L78S, L78D or L78F FNR_{ox} forms with WT Adx_{rd} led to spectral changes (data not shown) similar to those reported above for the reaction of Adx(4–108)_{rd} with WT FNR_{ox} (Fig. 5B), which are consistent with Adx_{rd} reoxidation. The time courses of these reactions presented k_{obs} values in the region of 0.01 s⁻¹ (Table 2). As all of these FNR forms have slightly less negative $E_{ox/sq}$ and $E_{sq/rd}$ values than the WT FNR (Table 2), it might be that their reduction by WT Adx becomes thermodynamically favoured.

Noticeably, the effects produced by the introduced mutations on FNR in the processes of FNR reduction by Adx_{rd} and Adx reduction by FNR_{rd} do not correlate with those reported for the corresponding reactions between FNR and Fd (Table 2) [8,13], suggesting that K75, L76, L78 and V136 are not critical in the Adx reduction by FNR.

Discussion

Differential spectroscopy analysis demonstrates that under our experimental conditions a 1 : 1 complex is formed between FNR_{ox} and both, Adx_{ox} as well as $Adx(4-108)_{ox}$. However, the K_d values obtained for such complexes indicate that they are considerably weaker than those reported for the [FNR_{ox}:Fd_{ox}] [10] and [AdR_{ox}:Adx_{ox}] [34] interactions (Table 1). Taking such evidence into account it is of interest to analyse if these complexes are produced in such an orientation that ET could take place within this hybrid system.

Stopped-flow and steady-state kinetic measurements indicate an ET process from FNR_{rd} to Adx_{ox} (Fig. 1) where Adx reduction is taking place. The reaction has been shown to occur with very low rate constants (Figs 2 and 3A), as compared with those reported for the physiological systems (Table 1) [10,34]. However, it is noticeable that, despite the high specificity that has been shown in the interactions between Fd and FNR and Adx and AdR, ET from FNR to Adx is also detectable. Thus, in both, Fd/FNR and Adx/AdR, systems it has been found that the single replacement of a residue can result in an important impairment of the optimal orientation for an efficient ET process [8,18,22]. Therefore, the very low ET rates obtained for the process between FNR_{rd} and Adx_{ox}, as compared with those of the physiological systems, can be easily understood by taking into account the lack of specificity at the FNR/Adx interface, which is known to be a main factor controlling ET reactivity [20,28,35]. Moreover, the initial phase shown in the kinetic traces (Fig. 1A, inset) and the ionic effect (Fig. 2C) observed when studying the ET reaction between FNR_{rd} and Adx_{ox} also suggest that in this hybrid system ET takes place after a minor reorganization of the initial transient complex has taken place [22,32].

The time-course for the reduction of Adx_{ox} by FNR_{rd} was found to fit to biphasic processes, with the exception of that at an Adx_{ox} :FNR_{rd} 1:1 ratio, with k_{obs} values decreasing with increasing Adx concentration, whereas the calculated V_0 values increase with Adx concentration. Such observations indicate that the two k_{obs} values might arise as the result of the presence of at least two different complexes for ET. Alternative modes of binding leading to different complexes between Adx and FNR, one of them being more suitable for ET, would not be unexpected due to the lack of specificity at the interface between these proteins. Such complexes have also been shown to appear upon replacement of a single FNR residue in the Fd/FNR system [35]. Moreover, the ability of Adx to form dimers, both in the crystalline state and in solution has been proposed [16]. These findings raise the question about its physiological significance and support the hypothesis of the existence of a ternary ET [Adx:Adx:AdR] complex in the physiological AdR/P450 system [16,36,37]. Therefore, our experimental data for the reduction of Adx by FNR_{rd} could fit a minimal mechanism:

is observed. However, when increasing Adx concentration, Adx reduction seems to occur following two different processes, where the amplitude (A_2) for the slower process (k_{obs2}) is larger than that (A_1) for the faster one (k_{obs1}) , suggesting that the faster process is limited by Adx concentration. These two different processes might account for those reactions stated above. Finally, the very slight biphasic dependence of k_{obs1} and V_0 on ionic strength suggests that whatever the complex involved, both, longrange electrostatic interactions for the initial protein-protein encounter and also short-range specific interactions at the protein-protein interface in the optimal complex for ET are rather weak. Nevertheless, our results clearly demonstrate that FNR is able to transfer electrons from NADPH to Adx through the formation of at least one productive transient complex. Furthermore, we have also proved that under steady-state conditions this NADPH/FNR/Adx ET system efficiently reduces a cytochrome P450 (i.e. CYP11A1). This result opens the door to using this system for the design of a multienzyme complex to make use of self-assembled monolayers of FNR coupled to gold electrodes [38], which will provide electrons for the reduction of different cytochrome P450 enzymes via the Adx carrier.

As Anabaena FNR is efficiently reduced by cyanobacterial Fd, it was also interesting to determine if Adx would sustain a similar ET reaction. As expected from the reduction potential values reported for Adx ($E_{WTAdx} =$ -270 mV) and FNR ($E_{\text{ox/rd}} = -320 \text{ mV}$, $E_{\text{ox/sq}} =$ -338 mV and $E_{sq/rd} = -312$), ET from Adx_{rd} to FNR_{ox} does not take place (Fig. 5A). However, when using a truncated Adx form [Adx(4-108)], which possesses a more negative reduction potential than the WT Adx $(E_{Adx(4-108)} = -344 \text{ mV})$ [23], reduction of FNR_{ox} (which is now a thermodynamically favoured process) is achieved (Fig. 5B). Nevertheless, in both the photosynthetic or steroidogenic systems we can find examples where nonthermodynamically favoured reactions take place upon complex formation [10,18,20]. In these cases shifts in the reduction potentials of the intermediate complex transition states have been related to the changes introduced in the redox cofactor environment upon complex formation. They are therefore related to the specificity and the strength of the protein-protein interaction. Therefore, the results presented here clearly indicate that although Adx_{rd} and FNR_{ox} can

$$Adx_{ox} + FNR_{rd} \longleftrightarrow [FNR_{rd} : Adx_{ox}] \xrightarrow{k_{ct}} [FNR_{sq} : Adx_{rd}]$$
(1)

$$[Adx_{ox}:Adx_{ox}] + FNR_{rd} \longleftrightarrow [FNR_d:Adx_{ox}:Adx_{ox}] \xrightarrow{k'_{ct}} [FNR_{sq}:Adx_{rd}:Adx_{ox}]$$
(2)

Because it has been reported that the equilibrium for dimer formation is shifted toward the dimer form when ionic strength is increased and toward the monomeric form when Adx concentration is increased [16], at very low Adx concentrations, dimerization of Adx will be favoured, resulting in Adx being reduced mainly through process Eqn (2). However, upon increasing Adx concentration reduction through both Eqns (1) and (2), processes would occur that are consistent with our observations. At an [Adx_{ox}]/[FNR_{rd}] ratio of 1, a very slow monophasic process achieve a correct orientation for ET, the interactions produced upon WT Adx_{rd} and FNR_{ox} binding are not strong enough to overcome the thermodynamic barrier for this ET process to proceed. It has also been shown that some residues on the FNR surface are essential for activity with Fd, either by providing an adequate interaction or by modulating the FAD reduction potential [8,13,31]. We have also tested if such residues determine the processes of FNR with Adx (Table 2) [8,13]. Our results clearly indicate that although some of the mutations in *Anabaena* FNR affect

the reactions between FNR and Adx slightly, probably due to the only small changes introduced in the reductase reduction potential values (Table 2), the effects produced neither correlate with the possibility of undergoing the ET processes analysed nor with those results reported for their reactions with Fd. Therefore, although K75 and the hydrophobic patch (L76 and L78) of FNR, crucial residues in the interaction with Fd [8,13], might modulate the FNR/ Adx interaction they are not critical for the ET processes. This result indicates that the FNR region critical for interaction with Fd is not determinant in the interaction with Adx, also suggesting that hydrophobic interactions might not be involved in FNR/Adx complex formation. In conclusion, our results clearly suggest that other mechanisms, unknown at this stage, are involved in determining the ability of this system to engage ET.

Although in the present study it is shown that the interaction observed between FNR and Adx allows ET from FNR to WT Adx and from Adx(4-108) to FNR, both ET processes are slow when compared with those in the physiological systems [10,34]. Structural comparison of the Adx(4-108) form with plant-type Fds has shown that, despite the low sequence identity, both types of structures are formed by a large core domain bearing the [2Fe-2S] centre and a smaller interaction domain [19]. Moreover, both Fd types are negative monopoles with a clear charge separation pointing to a region located in between the interaction domain and the [2Fe-2S] cluster. Thus, it is expected that in an initial approach the Adx negative monopole will focus the Adx [2Fe-2S] centre towards the Fd interaction domain of FNR, which is positively charged, as occurs in the physiological FNR/Fd and AdR/Adx interactions [5,18,28,39]. Our data clearly prove that such FNR/Adx interaction is taking place and that it might support ET. However, after this initial interaction between the two protein partners, reorganization of the complexes around the interaction surface has shown to take place in the FNR physiological system in order to achieve a more optimal orientation for ET [10]. Our results suggest that such reorganization is hardly taking place in the hybrid Adx/FNR system. Moreover, a comparative analysis of the interaction domain in both Fd types shows that it is structurally different in both subfamilies [19]. Therefore, as such reorganization has been shown to be induced by the formation of highly specific interactions among the surfaces of both protein partners [15], the large differences found in the interaction domains between Adx and Fd clearly explain why such productive interaction cannot be formed between Adx and FNR.

In conclusion, our results indicate that FNR and Adx are able to form productive complexes for ET, provided that the processes would be thermodynamically favoured. Moreover, mainly weak electrostatic long-range interactions must be involved in the formation of such complexes, which indicates a very low specificity of the interaction surface between FNR and Adx. As a consequence, the hybrid complexes obtained are not able to adopt orientations between the redox cofactors that would allow both ET rates as fast as those obtained with the physiological partners, and/or conformational changes and interactions that would overcome those nonthermodynamically favoured processes. However, the fact that ET is achieved in the Adx/FNR system supports the idea that the interaction between each reductase and the ET protein does not only take place through a highly specific complementarity of the protein surfaces and that other unknown mechanisms may also be involved in determining the ET ability of the system.

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