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Biophysical Chemistry 115 (2005) 219–224

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

Towards a new interaction enzyme:coenzyme

Marta Martínez-Júlvez, Jesús Tejero, José R. Peregrina, Isabel Nogués, Susana Frago, Carlos Gómez-Moreno, Milagros Medina*

Departamento de Bioquímica y Biología Molecular y Celular and Institute of Biocomputation and Physics of Complex Systems (BiFi), Facultad de Ciencias, Universidad de Zaragoza, 50009 Zaragoza, Spain

Received 3 June 2004; received in revised form 3 November 2004; accepted 10 December 2004
Available online 24 December 2004

Abstract

Ferredoxin–$NADP^+$ reductase catalyses $NADP^+$ reduction, being specific for $NADP^+$/H. To understand coenzyme specificity determinants and coenzyme specificity reversion, mutations at the $NADP^+$/H pyrophosphate binding and of the C-terminal regions have been simultaneously introduced in *Anabaena* FNR. The T155G/A160T/L263P/Y303S mutant was produced. The mutated enzyme presents similar k_{cat} values for NADPH and NADH, around 2.5 times slower than that reported for WT FNR with NADPH. Its K_m value for NADH decreased 20-fold with regard to WT FNR, whereas the K_m for NADPH remains similar. The combined effect is a much higher catalytic efficiency for NAD^+/H, with a minor decrease of that for $NADP^+$/H. In the mutated enzyme, the specificity for NADPH versus NADH has been decreased from 67,500 times to only 12 times, being unable to discriminate between both coenzymes. Additionally, giving the role stated for the C-terminal Tyr in FNR, its role in the energetics of the FAD binding has been analysed.

Keywords: Coenzyme specificity; $NAD(P)^+$/H; Ferredoxin–$NADP^+$ reductase

1. Introduction

The flavoenzyme ferredoxin–$NADP^+$ reductase (FNR, EC 1.18.1.2) catalyses the electron transfer (ET) from two reduced ferredoxin molecules to $NADP^+$, in order to produce NADPH, at the last step of the photosynthetic electron transfer chain [1–3]. This reaction is highly specific for $NADP^+$/H, with only a negligible activity with NAD^+/H [4]. However, the three-dimensional structure of FNR has been proposed to be the prototype of a family of flavin oxidoreductases dependent of either NAD^+/H or $NADP^+$/H [5,6], making FNR and its protein family a good model for coenzyme specificity redesign.

Nowadays, coenzyme specificity engineering is one of the main goals in enzyme redesign. Not strangely, the 2′-P or the 2′-OH interaction site of the coenzyme, as the only difference between the coenzymes $NADP^+$/H and NAD^+/H, respectively, has been the focus of initial redesign efforts [7–10]. Thus, in the case of *Anabaena* PCC7119 FNR, the role of several residues at 2′-P binding site of $NADP^+$/H, namely, Ser223, Arg224, Arg233 and Tyr235, has been stated [4]. Nevertheless, combinational mutation at these positions disrupted $NADP^+$/H binding, but was unable to further improve NAD^+/H-dependent activities [11]. It has been also shown that other regions of these enzymes might also contribute to coenzyme specificity, having been the focus of additional studies [7]. In the case of *Anabaena* FNR, involvement of the loops including residues 155–160 and 261–265 in binding of the coenzyme pyrophosphate moiety and determining coenzyme specificity was evident from mutational studies and structural analysis of the FNR:$NADP^+$ interaction [4,11,12]. In particular, a critical role for residues Thr155, Ala160 and Leu263 in *Anabaena* FNR has been proven. The simultaneous replacement of Thr155 by Ala, Ala160 by Thr and Leu263 by Pro, produced enzymes that were able to weakly bind NAD^+/H,

* Corresponding author. Tel.: +34 976 762476; fax: +34 976 762123.
E-mail address: mmedina@unizar.es (M. Medina).

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doi:10.1016/j.bpc.2004.12.034

suggesting that slightly different structural rearrangements of the backbone chain in the pyrophosphate binding region determine FNR coenzyme specificity [11]. The C-terminal Tyr of FNR, which forms a stacking interaction with the isoalloxazine moiety of FAD, has also shown to determine coenzyme specificity. Replacement of the C-terminal Tyr of pea FNR by Trp, Ser or Phe produced a decrease in the specificity towards $NADP^+$/H by drastically decreasing the ability of the enzyme to discriminate between $NADP^+$/H and NAD^+/H [12,13]. In fact, Y308S pea FNR has a k_{cat} value for the NADH-dependent activity that approached that of the NADPH-dependent activity of the WT enzyme, although it still has a relatively high K_m value [14]. This aromatic residue is found to be conserved in equivalent positions in many members of the FNR family that use $NADP^+$/H as preferred or exclusive coenzyme (Fig 1 in [14]). Moreover, this C-terminal residue is placed at the putative binding site of the nicotinamide moiety and far away from the 2′-P group of $NADP^+$/H. Analogous effects due to the mutation of the C-terminal residue have been described also in other $NADP^+$/H-dependent enzymes [9,15,16].

In the present study, mutations in the pyrophosphate and in the nicotinamide binding sites of *Anabaena* FNR have been combined trying to produce an FNR enzyme with increased specificity for NAD^+/H versus $NADP^+$/H. The mutated *Anabaena* FNR form T155G/A160T/L263P/Y303S has been produced and its spectral properties and kinetic parameters characterised. Additionally, and having into account the role played by the C-terminal Tyr in modulating the FAD reduction potential in *Anabaena* FNR [17], the energetic of the apoFNR:FAD binding for FNR mutants at the C-terminal position has been analysed.

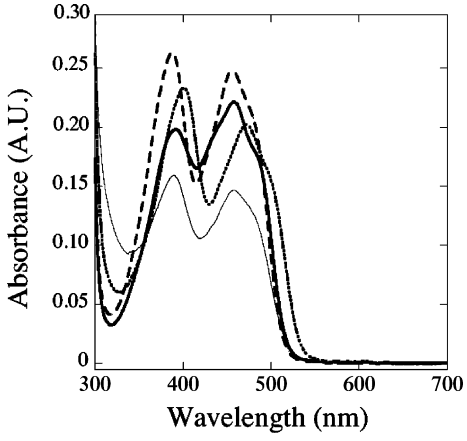

Fig. 1. Absorption spectra of WT (23.4 μM) (bold line), T155G/A160T/L263P/Y303S (26 μM) (broken line), T155G/A160T/L263P/Y303S (21.3 and 15 μM, respectively) in the presence of $NADP^+$ (27.3 μM) (dotted line) and in the presence of NAD^+ (950 μM) (thin line) *Anabaena* FNR forms in the visible region. The spectra were recorded in 50 mM Tris–HCl, pH 8.0 at 25 °C.

2. Experimental

2.1. Oligonucleotide-directed mutagenesis and protein production

The T155G/A160T/L263P/Y303S *Anabaena* FNR mutant was produced using the QuikChange mutagenesis kit (Stratagene). The Y303S mutation was added to the sequence of the T155A/A160T/L263P pET28-FNR vector previously produced [11]. The mutated FNR fragment was transformed into *Escherichia coli* BL21 (DE3) Gold cells (Stratagene). The mutant was purified from LB cell cultures as previously described [11,18]. UV–visible spectra and SDS–polyacrylamide gel electrophoresis were used as purity criteria.

2.2. Spectral analysis and enzymatic assays

UV–visible spectral analyses were carried on a Kontron Uvikon 942 spectrophotometer. FNR diaphorase activity with DCPIP as artificial electron acceptor was determined as described previously [4]. Both NADPH and NADH were assayed as coenzyme electron donors to FNR in 50 mM Tris–HCl, pH 8.0 at 25°C. In all measurements, direct reduction of DCPIP by the coenzyme was subtracted from that of the enzyme–coenzyme mixture. The kinetic results obtained from the diaphorase activity were interpreted using the Michaelis–Menten kinetic model.

2.3. Binding energy differences of FNR mutants to FAD upon reduction

Determination of the differences between the free energies for the [apoFNR:FAD] complexes in the different redox states ($\Delta\Delta G$) for WT, Y303W, Y303F and Y303S *Anabaena* FNRs was made using Eqs. (1) (2) (3):

$$\Delta\Delta G_{sq-ox} = \Delta G_{sq} - \Delta G_{ox} = -F\left(E_{ox/sq} - E^{free}_{ox/sq}\right) \quad (1)$$

$$\Delta\Delta G_{rd-sq} = \Delta G_{rd} - \Delta G_{sq} = -F\left(E_{sq/rd} - E^{free}_{sq/rd}\right) \quad (2)$$

$$\Delta\Delta G_{rd-ox} = \Delta G_{rd} - \Delta G_{ox} = -F\left(E_{ox/sq} - E^{free}_{ox/sq} + E_{sq/rd} - E^{free}_{sq/rd}\right) \quad (3)$$

where $\Delta\Delta G$ values correspond to the oxidised, one-electron reduced and two-electron reduced holoprotein complexes and $E^{free}_{ox/sq}$ and $E^{free}_{sq/rd}$ are the reduction potentials of free FAD for the ox/sq (oxidised/semiquinone) and sq/rd (semiquinone/reduced) couples. The values for $E_{ox/sq}$ and $E_{sq/rd}$ were taken from Ref. [17].

3. Results

3.1. Expression and purification of T155G/A160T/L263P/Y303S FNR

The level of expression of the mutant form was similar to that of the recombinant WT enzyme. During purification, the visible spectrum of mutated FNR suggested that part of preparation consisted of $NADP^+$-bound enzyme, as already reported for some *Anabaena* and pea FNR mutants at the residue equivalent to Tyr303 [13, 17]. Chromatography on a Cibacron-blue matrix efficiently removed the nucleotide from FNR molecules.

3.2. Spectral properties

Simultaneous replacements of Thr155 by Gly, Ala160 by Thr, Leu263 by Pro and Tyr303 by Ser in *Anabaena* FNR resulted in an enzyme with alterations in the UV–Vis spectra of the bound flavin (Table 1, Fig. 1). The absorbance maxima values for transition bands I and II of T155G/A160T/L263P/Y303S FNR were slightly shifted to shorter wavelengths relative to the WT FNR values (1–4 nm). Not only the positions of maxima values, but also the intensity ratio between both transition bands was altered by the introduced mutations. Thus, WT FNR shows lower intensity at band II than at band I, whereas the opposite effect is shown by the mutated FNR (Table 1, Fig. 1). Furthermore, the shoulder shown by WT FNR at 480 nm is almost lost in the spectrum of T155G/A160T/L263P/Y303S FNR (Fig. 1). These observations clearly indicate perturbation in the FAD microenvironment by the introduced mutations, which is in agreement with previous structural data showing that replacement of the FNR C-terminal Tyr by Ser increased the exposure of FAD to the solvent [13].

Coenzyme binding also produces alteration of the mutated enzyme spectrum. Thus, addition of $NADP^+$ considerably shifts transition bands II and I to longer wavelengths and also alters the absorbance ratio II/I with regard to the free mutant (Fig. 1, Table 1). Such important shifts are not observed upon addition of $NADP^+$ to WT FNR by regular absorbance spectroscopy. In the WT case very small spectral perturbations can be only observed by using differential spectroscopy [4,19]. Although addition of NAD^+ is also unable to produce any perturbation in the WT FNR spectrum, its addition to T155G/A160T/L263P/Y303S FNR produces small shifts of both transition bands as detected by regular absorbance spectroscopy (Table 1, Fig. 1).

Table 1
UV–Vis spectral properties of WT and mutated *Anabaena* PCC 7119 FNR_{ox} Forms[a]

FNR form	UV maximum (nm)	Band II maximum (nm)	Band I maximum (nm)	Abs ratio II/I
WT	274	391	458	0.89
T155G/A160T/L263P/Y303S	273	387	455	1.06
T155G/A160T/L263P/Y303S-$NADP^+$[b]	271	401	472	1.16
T155G/A160T/L263P/Y303S-NAD^+[c]	279	389	458	1.09

[a] All spectra were recorded in 50 mM Tris–HCl, pH 8.0, at 25 °C.
[b] Recorded in the presence of $NADP^+$(27.3 μM) with FNR mutant 21.3 μM.
[c] Recorded in the presence of NAD^+(950 μM) with FNR mutant 15 μM.

3.3. Kinetic parameters of T155G/A160T/L263P/Y303S FNR

The steady-state kinetic parameters of the mutated FNR form for the diaphorase activity with DCPIP were determined using either NADPH or NADH as electron donor (Table 2). Previous analysis of the T155G/A160T/L263P FNR showed that this mutant maintains a k_{cat} value with NADPH comparable to that of the WT FNR while exhibiting just a slight increment of its K_m value [11]. As a consequence of these combined effects a twofold decrease of its catalytic efficiency was observed (Table 2). In the present work we show that introduction of the additional mutation, Y303S, in the T155G/A160T/L263P FNR mutant produces an enzyme, T155G/A160T/L263P/Y303S FNR, that exhibits a K_m value similar to that of WT enzyme and a twofold decrease of the k_{cat} value (Table 2). Thus, the new produced mutant shows a catalytic efficiency with NADPH in the same order of magnitude of that exhibited for WT FNR. The effect of the single substitution of the C-terminal Tyr by Ser has been reported in the case of pea FNR (Y308S mutant in pea, 303 residue in *Anabaena*) by following the enzyme diaphorase activity with ferricyanide as electron acceptor (Table 2) [14]. Although the k_{cat} value reported for the Y308S pea FNR in the reaction with NADPH was 15-folds lower than that exhibited by WT FNR, a 55-fold decrease of the K_m value for NADPH was also observed, which produced an enzyme 3.5 times more efficient than the WT pea FNR [14]. Thus, T155G/A160T/L263P/Y303S FNR apparently combines the effects of the Y303S and the T155G/A160T/L263P mutants, being clearly patent that the residues at the pyrophosphate bridge modulate the strong affinity for $NADP^+$ introduced upon replacement of the C-terminal Tyr by a Ser.

Analysis of the kinetic parameters of T155G/A160T/L263P/Y303S FNR when using NADH as electron donor in the DCPIP diaphorase assay indicates a considerably increment in the affinity for NADH with regard to the WT enzyme, as judged by the important decrease in the K_m value (by 20-fold), and a considerably increase in the k_{cat} value (by 194-folds) (Table 2). Thus, the overall effect shown by this mutant is a catalytic efficiency with NADH increased by a factor of 4000 times with regard to that of WT FNR. Additionally, comparison of these kinetic parameters with those previously reported for the T155G/

Table 2
Steady-state kinetic parameters for the FNR diaphorase activity with DCPIP of WT and mutated *Anabaena* FNR forms[a]

FNR forms	NADPH			NADH			Specificity for NADPH
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	
Anabaena WT	6[b]	81.5[b]	13.5[b]	800[c]	0.16[c]	2×10^{-4}[c]	67,500
T155G/A160T/L263P/Y303S	4.1	38.5	9.4	39.4	31	0.8	11.75
T155G/A160T/L263P[d]	12	77	6.4	390	0.33	8.4×10^{-4}	7,619
Pea WT[e]	24	490	20	16,000	8.8	5.5×10^{-4}	36,000
Pea Y308S[e]	0.44	32	73	450	430	0.95	77

[a] All the activities were measured in Tris–HCl 50 mM, pH 8.0 at 25 °C.
[b] Data from Ref. [18].
[c] Data from Ref. [4].
[d] Data from Ref. [11].
[e] Data from Ref. [14].
Data reported for diaphorase assays with $K_3Fe(CN)_6$.

A160T/L263P FNR mutant indicates that introduction of the Y303S mutation on the triple mutant enhances the catalytic efficiency by a factor of 950 by improving both electron transfer and coenzyme interaction.

In consequence, introduction of the Ser at position 303 of *Anabaena* T155G/A160T/L263P FNR considerably increases the catalytic efficiency for NADH while almost no changes are produced in that value for NADPH. Thus, the overall effect is that the T155G/A160T/L263P/Y303S FNR mutant shows a specificity for NADPH versus NADH of only 12-fold, a value considerably low when compared with the 7620-fold in T155G/A160T/L263P FNR or the 67,500 in WT FNR (Table 2). It is also worth noting that the catalytic efficiency of the T155G/A160T/L263P/Y303S FNR with NADH has considerably approached to that of WT FNR with NADPH (Table 2).

3.4. Role of C-terminal Tyr in FAD binding

Due to the irreversible denaturation of *Anabaena* FNR upon FAD dissociation from the holoprotein, it is difficult to experimentally determine absolute values for the K_d and binding energies for the [apoFNR:FAD] complex in its different redox states [20]. However, since the flavin reduction potentials are related to the affinity of the apoFNR:FAD interaction in different FAD reduction states by thermodynamic cycles, it is possible to calculate differences between the binding energies for the oxidised, one-electron reduced and two-electron reduced holoprotein complexes [21]. Thus, the $\Delta\Delta G_{sq\text{-}ox}$, $\Delta\Delta G_{rd\text{-}sq}$ and $\Delta\Delta G_{rd\text{-}ox}$ values were determined for WT, Y303F, Y303S and Y303W FNR variants using the values reported for their reduction potentials [17] and Eqs. (1) (2) (3) (see Experimental) (Table 3). Fig. 2 shows how the differences in free energies vary as the complex becomes reduced for each FNR form. Complexes of apoFNR with reduced FAD are considerably less stable than those of the semiquinone or oxidised states for most of the FNR forms. This might explain why reductases from various species undergo rapid inactivation when maintained in the reduced state [3]. Since the WT FNR and the Y303W FNR variant display identical reduction potential values [21], they also exhibit identical profiles, whereas replacement of Tyr303 by Phe only slightly changes this energy profile. Thus, the apoFNR form of this mutant binds to the FAD semiquinone more tightly than to the oxidised one, whereas the magnitude of the destabilisation observed upon binding to the reduced FAD relative to the oxidised cofactor is up to 1 kcal/mol smaller than that found for the WT FNR complexes. However, the profile for Y303S FNR is completely different from the others (Fig. 2). It has been reported that this mutant shows an almost lack of stabilisation of semiquinone state and its reduction potential

Table 3
Differences in binding energies of the oxidized, semireduced and reduced ApoFNR: FAD complexes of WT and mutated *Anabaena* FNR forms

FNR Form	$E_{ox\text{-}rd}$[a] (mV)	$E_{ox\text{-}sq}$[a] (mV)	$E_{sq\text{-}rd}$[a] (mV)	$\Delta\Delta G_{sq\text{-}ox}$[b] (kcal mol^{-1})	$\Delta\Delta G_{rd\text{-}sq}$[b] (kcal mol^{-1})	$\Delta\Delta G_{rd\text{-}ox}$[b] (kcal mol^{-1})
WT	−374	−385	−371	0.276	6.026	6.302
Y303F	−356	−358	−354	−0.345	5.635	5.29
Y303S	−250	−338	−162	−0.805	1.219	0.414
Y303W	−376	−383	−369	0.23	5.98	6.21

[a] Data from Ref. [17].
[b] Values calculated by using Eqs. (1) (2) (3) (see Experimental).

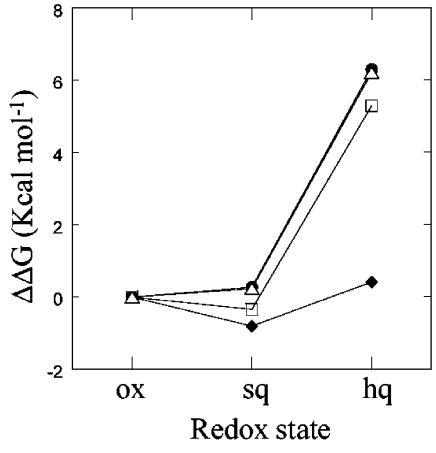

Fig. 2. Binding energy profiles of WT ApoFNR (filled circles), Y303F ApoFNR (open squares), Y303S ApoFNR (filled rhombi) and Y303W ApoFNR (open triangles) to FAD in different redox states.

values are much closer to the potentials of the free flavin than those of the other FNR variants [17]. Its profile indicates that complex of apoFNR with reduced FAD is only slightly less stable than those of semiquinone or oxidised states. In any case, differences of binding energies for Y3030S FNR are much smaller than those for the other FNR variants, indicating that complexes of [apoY303S:-FAD] are almost equally stable in any redox potential state of the FAD cofactor.

4. Discussion

Important structural differences have been reported for the L263P, T155G/A160T and T155G/A160T/L263P mutated FNR forms in the loop comprising residues 261–265 [11]. Thus, simultaneous introduction at positions Thr155, Ala160 and Leu263 of residues usually found in NAD^+/H-dependent reductases produced an enzyme, T155G/A160T/L263P FNR, with a conformation for the 261–265 loop resembling those found in the NAD^+/H-dependent members of the FNR family. Such observations, together with previous studies in the pea FNR indicating that the C-terminal Tyr308 (Tyr303 in *Anabaena*) was also a determinant of coenzyme specificity [14], prompted us to probe the effect of simultaneous substitutions at positions 155, 160, 263 and 303 of *Anabaena* FNR.

Upon introduction of the Y303S mutation on the T155G/A160T/L263P FNR (which spectrum was indistinguishable from that of WT FNR [11]), important alterations of the UV–Vis spectra were observed (Fig. 1, Table 1). As the visible spectrum is only related to the FAD prosthetic group, this indicates a modification of the isoalloxazine ring microenvironment. A similar effect was already described upon single replacement of the C-terminal Tyr by a Ser in *Anabaena* and pea FNRs [14,17]. Additionally, important spectral changes are produced in the T155G/A160T/L263P/Y303S FNR spectrum when recorded in the presence of NAD^+, and specially $NADP^+$, suggesting that this mutant binds both coenzymes, and that upon coenzyme binding the isoalloxazine ring environment results altered in a larger extent than in the WT or T155G/A160T/L263P FNRs.

Steady-state kinetic parameters of the T155G/A160T/L263P/Y303S FNR mutant clearly indicate that the addition of the Y303S mutation to the T155G/A160T/L263P FNR mutant produces an enzyme that considerably increases the efficiency with NADH (making the enzyme up to 4000-fold more efficient with NADH than the WT) by simultaneously increasing the enzyme affinity for the coenzyme and by improving the hydride transfer process itself. However, the catalytic efficiency of the T155G/A160T/L263P/Y303S FNR for NADPH remains in a similar range of that of WT. Although almost no alteration of the K_m value for NADPH is observed with regard to WT, a slight decrease of the catalytic rate is observed (Table 2). Such observations suggests that binding of NADPH to the T155G/A160T/L263P/Y303S FNR might produce a less efficient orientation for hydride transfer between the isoalloxazine ring of FAD and the nicotinamide moiety of NADPH than that of the WT enzyme. However, these changes might also be related to a slow release of the products as reported for the pea Y308S mutant [14]. Thus, the kinetic parameters of the T155G/A160T/L263P/Y303S mutant indicate that combination of these four mutations produces an enzyme only 12 times more specific for NADPH than for NADH (Table 2). Therefore, in this mutant specificity for NADPH has been considerably decreased as compared to the values exhibited by WT (67,500-fold) and T155G/A160T/L263P/Y303S (7620-fold) FNRs. This behaviour of T155G/A160T/L263P/Y303S FNR mutant shows some similarities with that previously reported for the Y308S pea. The Y308S mutant showed a considerably increase in the affinity for both NADPH and NADH along with an important increment in the k_{cat} for NADH. These changes yielded an enzyme that was only 77 times more specific for NADPH than for NADH [14]. Nevertheless, the high affinity of the pea Y308S FNR by NADPH might affect the product release rate being probably the reason of the low turn-number exhibited by this mutant (Table 2). In conclusion, mutations combining residues Thr155, Ala160 and Leu263 at the pyrophosphate binding site of $NADP^+$/H in FNR with the introduction of a Ser at the C-terminal position of *Anabaena* FNR produce an enzyme which can function with NAD^+/H almost as efficiently as with $NADP^+$/H, therefore breaking the high specificity shown by WT FNR for $NADP^+$/H. These studies also bring us one step further to our final goal, the reversion of coenzyme specificity in FNR.

Having into account the important role played by the C-terminal Tyr, not only in the catalytic mechanism itself, but also in modulating the redox properties of the flavin ring within the FNR structure [17], analysis of several mutations at this position (Phe, Trp and Ser) in the energetic of the FAD binding in its different redox states has been carried out (Table 3). Only introduction of a Ser at position Tyr303 of *Anabaena* FNR considerably changes the binding energy profile of FAD in its three redox states (Fig. 2). Thus, the [apoY303S FNR:FAD] complex does not show destabilisation upon FAD reduction, effect exhibited when either a Tyr, Phe or Trp are placed at the C-terminal position. However, since we are not able to calculate binding parameters but only differences in cofactor affinity between the different redox states, we do not know how strong the formed complexes between apoY303S FNR and FAD are in comparison with the other FNR forms. Nevertheless, taking into account that the destabilisation of the [apoFNR:FAD] complex during FAD reduction in the case of WT FNR appears to result from an electrostatic repulsion between the anionic hydroquinone, with a negative charge at N(1) of the flavin ring, and some neighbouring negatively charged residues [18], the smaller extent of destabilisation showed by [apoY303S FNR:FAD] complexes during cofactor

reduction could be explained by the larger exposition of FAD to the solvent in this structure, which might compensate the flavin ring charged introduced upon reduction by charge-dipole interactions. Thus, the data here reported clearly state that Tyr303 modulates the protein/flavin interaction.

Acknowledgements

This work has been supported by Comisión Interministerial de Ciencia y Tecnología (CICYT and BIO2003-00627 to C. G.-M. and Grant BIO2004-00279 to M.M.).

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