Elsevier Editorial System(tm) for Archives of Biochemistry and Biophysics

Manuscript Draft

Manuscript Number: ABBI-07-950R1

Title: Structural and functional diversity of ferredoxin-NADP+ reductases

Article Type: Special Issue: Enzymology in Europe (PF)

Section/Category: Protein Biochemistry and Biophysics

Keywords: Flavoprotein; flavin; NADP; photosynthesis; induced fit; electron transfer; Apicomplexa; Plasmodium falciparum; malaria

Corresponding Author: Prof. Alessandro Aliverti, Ph.D.

Corresponding Author's Institution: Universita' degli Studi di Milano

First Author: Alessandro Aliverti, Ph.D.

Order of Authors: Alessandro Aliverti, Ph.D.; Vittorio Pandini; Andrea Pennati; Matteo de Rosa; Giuliana Zanetti

Manuscript Region of Origin:

Abstract: Although all ferredoxin-NADP+ reductases (FNRs) catalyze the same reaction. i. e. the transfer of reducing equivalents between NADP(H) and ferredoxin, they belong to two unrelated families of proteins: the plant type and the glutathione reductase type of FNRs. Aim of this review is to provide a general classification scheme for these enzymes, to be used as a framework for the comparison of their properties. Furthermore, we report on some recent findings, which significantly increased the understanding of the structure-function relationships of FNRs, i.e. the ability of adrenodoxin reductase and its homologs to catalyze the oxidation of NADP+ to its 4-oxo derivative, and the properties of plant-type FNRs from non-photosynthetic organisms. Plant-type FNRs from bacteria and Apicomplexan parasites provide examples of novel ways of FAD and NADP(H) binding. The recent characterization of an FNR from Plasmodium falciparum brings these enzymes into the field of drug design.

UNIVERSITÀ DEGLI STUDI DI MILANO
DIPARTIMENTO DI SCIENZE BIOMOLECOLARI E BIOTECNOLOGIE

D S B B

Milano, February 6th, 2008

To the Editor
of Archives of Biochemistry and Biophysics

Subject: revision of ms ABBI-07-950

Dear Editor,

we would like to thank the Reviewers for their criticisms and suggestions that hopefully allowed us to improve our article. We carefully revised the manuscript according to the Reviewer's suggestions. The overall length of the paper has been significantly reduced by shortening longer chapters and by deleting three figures.

The major changes in the revised version of the manuscript are as follows:

Reviewer 1.

1) The authors strive to avoid confusion and refer, rightfully, to the ferredoxin reductase-type enzymes as the plant-type FNRs. A similar approach should be used to refer to the other subclass of FNRs, which is presented initially as the glutathione reductase-type class and referred to later on in the paper as the adrenodoxin reductase-type class. The presentation would gain in clarity if the second subclass would be defined as the adrenodoxin reductase-type right on in the introduction.
"GR-type" and "AdR-like" are not synonymous. Indeed, as shown in Fig. 1, GR-type FNRs represent a broader group that include both the AdR-like and the ONFR-like enzymes. Thus, we prefer to maintain the distinction between the two terms (GR-type and AdR-like).

2) While most of the chapters have similar lengths, the last two chapters are significantly longer than the previous six ones, creating an unpleasant imbalance in the presentation. The authors should try to either condense or split in multiple chapters the two long ones, in order to have a more armonious and balanced presentation of the topics.
Large chapters ("Specific features of Plasmodium falciparum FNR" and "Ferredoxin binding and electron-transfer") were significantly shortened (by 35% and 22%, respectively). Previous very long paragraphs were broken into smaller ones.

3) The work would significantly gain in strength upon adding a small summary paragraph at the end of the review article. Alternatively, the authors may consider a paragraph in which they present open questions that can now be raised and answered based upon current knowledge.
A Conclusion chapter has been added. However, in order to limit the ms length, this chapter is not a summary or a list of open questions, but includes just some concluding remarks.

Via Celoria, 26 – 20133 MILANO (Italia)
Telefono 02/50315042 Fax 02/50315044 e-mail: segregen.dbsb@unimi.it

4) References to the literature should be properly added to backup several statements that are made. For example, and by no means being a comprehensive list: on page 3 lines 3 and 8; page 4 line 2; page 7 line 7; page 8 line 4 from the bottom; page 13 line 20; page 14 lines 9, 11, 14 and 17.
Several citations have been added throughout the text, in particular at the sites indicated by the Reviewer.

5) A list of abbreviations should be included. For example, what is NMN?
A list of abbreviations has been added.

6) On page 4 line 15, both n and S should be defined.
The definition of the parameters n and S has been given, and a reference added.

7) The authors should specify clearly which enzymes have been wrongly identified as adrenodoxin reductase-like enzymes (page 6).
What we found is that some AdR-like proteins have been wrongly identified as other enzymes, and not the opposite. We think that, for the sake of brevity, it is not possible to give a comprehensive list of incorrectly identified entries within this review article. We have provided just one example of such observation.

8) Several grammar mistakes and typos are present throughtout the manuscript, which will have to be corrected.
We tried to fix all mistakes and typos.

Reviewer #2:
In general, I think the readability would be improved if the authors were to shorten it by about 25%+ by removing details that distract from the overall message and flow, and that interested readers can get from the original literature. Also, sometimes long paragraphs are very difficult to process and retain information from. For instance, the "NADP binding" is a single long paragraph, the "plasmodial FNR" section has a 2 page paragraph and the "ferredoxin binding and electron transfer" section appears to be a single 3 page paragraph. These must be broken up into more digestable units with clear points to be gotten.
The "plasmodial FNR" chapter has been radically shortened by removing most details (see reply to Reviewer #1, point 2) Three figures have been deleted. Other parts of the ms have been shortened as well, although at a lower extent.

-p3:Having the single name ferredoxin reductase cover what turned out to be two distinct enzyme types has long been confusing and this manuscript can help clarify their relationships, but it must be careful to not add to the confusion. The diagram in figure 1 is good, but I suggest adding coloring or wording to make clear that all the plant FNR's are one homologous family and all the GR-like FNRs are a distinct family.

Fig. 1 and its legend have been modified in order to make the point raised by the Reviewer clear.
To avoid very confusion, it is crucial that the language in the introduction and throughout be very clear: e.g. the sentence on p. 3 "FNRs do not represent a homogeneous group of proteins," should be replaced with the more direct "FNRs can be grouped into two phylogenetic/structural families that we here refer to as the "plant type" and "GR-like" FNRs (Figure 1)."; the term "subclasses" later used on p. 3 needs to be replaced with something like "evolutionary families." Also p. 3, where the reviews on plant-type FNRs are cited, reviews for GR-like FNRs should also be cited so up front both families are well introduced. One other thing that I think would help the reader is if the authors explicitly noted right at the start that the nomenclature of having both enzyme families sometimes called FNR and other times only one of the families (plant-type) referred to as FNR has led to much confusion.

The Introduction and the following chapter were modified as suggested by Reviewer #2.

-p.4: the sentences "All FNRs have a similar gross structure" and "despite this apparent similarity ". Since the folds of the FAD binding domains are totally distinct even at a glance, it is very misleading to say they have an apparent similarity that leads it to be surprising they cluster in different phylogenetic groups. The section could be most clearly written simply starting with the two groups that were already introduced in the intro and mentioning from their how sequence comparisons further break them down.
The text has been revised as suggested.

-p.5: AdR also has FAD and NADP domains with similar topology, so I don't see how it is different from ONFR in that regard?
The misleading term “topology” has been changed to organization. What we meant is that ONFR has both domains sharing a β/β/α layered structure, whereas AdR has the FAD- and the NADP-binding domains adopting a β/β/α and a α /β/α organization, respectively.

p. 11: the time and figures spent on the dimerization of plasmodial FNR is too much given its treatment in the original literature and the lack of evidence it is anything more than a crystallization artifact. I'd suggest deleting figure 9 and 10.
The figures have been deleted.

Fig. 3 - The scale and orientation of panel C should match the scale and orientation of panel D to maximize the readers ability to see the structural similarity.
Panels C and D of Fig. 2 have been redrawn according to the Reviewer’s suggestions.

Fig. 8 is unnecessary. The simple one sentence point that there are fewer charges at the 2'-phosphate binding site does not need a figure to illustrate it.
The figure has been deleted.

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We hope that the current revised version of our manuscript is amenable for publication in ABB.

Yours, sincerely

Alessandro Aliverti

Via Celoria, 26 – 20133 MILANO (Italia)
Telefono 02/50315042 Fax 02/50315044 e-mail: segregen.dbsb@unimi.it

Text with changes Marked

Structural and functional diversity of ferredoxin-NADP$^+$ reductases

Alessandro Aliverti*, Vittorio Pandini, Andrea Pennati[1], Matteo de Rosa, and Giuliana Zanetti

Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano, via Celoria 26, 20133 Milano, Italy.

[1]Current Address: Department of Chemistry, Georgia State University, Atlanta, GA 30302-4098, U.S.A.

Short title: Ferredoxin-NADP$^+$ reductases

*To which correspondence should be addressed at: Alessandro Aliverti, Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano, via Celoria 26, 20133 Milano, Italy, tel. +39 02 503 14897, fax +39 02 503 14895, e-mail alessandro.aliverti@unimi.it

Abstract

Although all ferredoxin-$NADP^+$ reductases (FNRs) catalyze the same reaction. *i. e.* the transfer of reducing equivalents between NADP(H) and ferredoxin, they belong to two unrelated families of proteins: the plant type and the glutathione reductase type of FNRs. Aim of this review is to provide a general classification scheme for these enzymes, to be used as a framework for the comparison of their properties. Furthermore, we report on some recent findings, which significantly increased the understanding of the structure-function relationships of FNRs, *i.e.* the ability of adrenodoxin reductase and its homologs to catalyze the oxidation of $NADP^+$ to its 4-oxo derivative, and the properties of plant-type FNRs from non-photosynthetic organisms. Plant-type FNRs from bacteria and Apicomplexan parasites provide examples of novel ways of FAD and NADP(H) binding. The recent characterization of an FNR from *Plasmodium falciparum* brings these enzymes into the field of drug design.

Keywords: Flavoprotein, FAD, NADP, photosynthesis, induced fit, electron transfer, Apicomplexa, *Plasmodium falciparum*, malaria

Abbreviations

AdR, adrenodoxin reductase; Adx, adrenodoxin; FNR, ferredoxin:$NADP^+$ oxidoreductase; Fd, ferredoxin; GR, glutathione reductase; NADPO, 4-oxo-NADP; NMN; nicotinamide mononucleotide; 2'-P-AMP, 2'-phospho-AMP.

Introduction

Ferredoxin:$NADP^+$ oxidoreductases (FNRs, EC 1.18.1.2) are FAD-containing flavoenzymes that catalyze the transfer of reducing equivalents between ferredoxins (Fds) and the $NADP^+$/NADPH couple according to the following equation [1]:

$$2Fd_{red} + NADP^+ = 2Fd_{ox} + NADPH \qquad \text{Eq. 1}$$

In several different organisms and tissues, FNRs participate in electron transfer chains involved in metabolic processes as diverse as photosynthesis, nitrogen fixation, isoprenoid biosynthesis, steroid metabolism, xenobiotic detoxification, oxidative-stress response, and iron-sulfur cluster biogenesis [1-5]. Eq. 1 represents the electron flow trough FNR as it occurs in the photosynthetic electron chain. However, the physiological direction of the reaction catalyzed by FNRs involved in the other pathways is opposite, *i. e.* toward the production of reduced Fd. On this basis, FNRs are sometimes classified as autotrophic (photosynthetic FNRs) and heterotrophic (all other FNRs) [6]. FNRs can be grouped into two phylogenetic/structural families that we here refer to as plant-type and glutathione reductase (GR)-type FNRs (Fig. 1). It should be noted that the term FNR is inconsistently used by different authors to denote either exclusively the plant-type FNRs or both enzyme families, thus bringing much confusion. A huge number of papers have been published on FNRs since the discovery of photosynthetic FNR and mitochondrial adrenodoxin reductase (AdR) in the sixties. Excellent reviews on plant-type FNRs appeared in recent years, and the reader is referred to them for an introduction to the field [1, 4, 5, 7]. To the best of our knowledge, no reviews on the GR-type FNRs have been published so far. The only article about the general properties of AdR appeared more than 15 years ago [8]. The scope of the present review is to cover aspects not adequately considered in previous work, with a special focus on two topics. First, we will provide a comparison between plant-type and GR-type FNRs. Second, we will summarize the most recent findings on these two highly important enzyme families.

Structural and phylogenetic classification of FNRs

As anticipated in the Introduction, enzymes that catalyze the reaction described in Eq. 1, and thus referred to as FNRs, belong to two phylogenetically and structurally unrelated protein families. The sequences of several hundred proteins annotated as FNR have been deposited in the SwissProt and GenBank NCBI databases. More than 50 entries dealing with the crystal structures of FNRs are also currently available in the Protein Data Bank. By comparison of their three-dimensional structures and sequences the two FNR families have been further subdivided as outlined in Fig. 1. Thus plant-type FNRs comprise the plastidic and bacterial enzymes, whereas the AdR-like flavoproteins and, for the reasons detailed below, the ONFRs belong to the GR-type FNRs. FNRs of both families possess a two-domain organization with the active site located at the interface between the FAD- and the NADP-binding domains (Fig. 2). Dym and Eisenberg have identified 4 general folds for FAD binding in 32 families of FAD-containing proteins [9]. The FAD-binding domain of the GR-type and the plant-type FNRs adopt two of such general folds.

The above subdivision of FNRs in two evolutionary families is in agreement with both CATH (http://www.cathdb.info) and SCOP (http://scop.berkeley.edu) classifications of protein domains [10, 11]. The FAD-binding domain of plant-type FNRs is based on an antiparallel β-barrel with a greek-key topology (number of strand, $n = 6$, and shear number, $S = 10$) [12]. The corresponding domain of AdR-like FNRs adopts a three-layer β/β/α fold, with a central parallel five-membered β-sheet of 32145 topology. It should be noted that, while in plant-type FNRs the FAD-binding domain is formed by the N-terminal portion of the polypeptide chain, in GR-type enzymes two discontinuous segments of the polypeptide form the FAD-binding domain (Fig. 2). The NADP-binding domain of both FNR families is a three-layer sandwich α/β/α, with a Rossmann-like topology and a parallel 5-membered β-sheet. However, these domains differ in several details in the two families of FNRs, particularly in the precise mode in which NADP(H) is bound.

It is worth to mention that a family of flavoproteins exists, which, although NAD-dependent, are clearly structurally and functionally related to the AdR-like FNRs. This family includes the bacterial oxygenase-coupled NADH-ferredoxin reductases (ONFRs), of which the *Pseudomonas* ferredoxin reductase component of biphenyl dioxygenase (BphA4) is the best characterized member [13]. ONFR-like enzymes (EC 1.18.1.3), though not formally FNRs, will be shortly dealt with in this review, since their properties help in understanding the structure-function relationships of AdR-like proteins. ONFRs-like enzymes mainly differ from AdR-like FNRs in having both FAD- and NAD-domain sharing the same organization (a three-layer β/β/α fold related to the FAD-binding domain of AdR) and in possessing a third C-terminal domain involved in protein homodimerization (Fig. 2).

Finally, it is interesting to note that both families of FNRs are part of wider protein superfamilies that include non-FNR members. The "FNR superfamily" consists of proteins that share the two-domain unit of plant-type FNR and possess a variety of additional domains [7, 14, 15]. It comprises nitrate reductase, phthalate dioxygenase reductase, sulfite reductase and the dual flavin oxidoreductases such as cytochrome P450 reductase, NO synthase and methionine synthase reductase. Similarly, AdR-like and ONFR-like enzymes belong to the "two dinucleotide binding domains" flavoprotein superfamily (tDBDF) [16], which includes the Baeyer-Villiger monooxygenase, glutamate synthase β subunit, dehydropyridine dehydrogenase, thioredoxin reductase, and glutathione reductase.

Plant-type FNRs have been studied extensively, with the functional and structural characterization of enzymes from several eukaryotic and prokaryotic organisms. In particular, high resolution three-dimensional structures have been solved for the FNRs from spinach (*Spinacia oleracea*) leaf [17], corn (*Zea mays*) leaf and root [18, 19], pea (*Pisum sativum*) leaf [20], paprika (*Capsicum annuum*) leaf [21], the protozoon *Plasmodium falciparum* [22], the cyanobacteria *Anabaena* [23, 24] and *Synecochococcus* (PDB accession no. **2B5O**), and the bacteria *Escherichia coli* [25], *Azotobacter vinelandii* [26] and *Rhodobacter capsulatus* [27]. The 3D structure and the

amino acid sequences of these proteins have been compared in detail leading to the definition of the consensus of six sequence motifs highly conserved in plant-type FNRs [1] and to the identification of the groups in which these enzymes can be further subdivided (Fig. 1), each characterized by specific structural features [1, 7].

Conserved structural features in AdR-like FNRs

As mentioned before, AdR-like FNRs have been studied to a lower extent than the plant-type ones. For instance, three-dimensional structures have been obtained only for bovine AdR [28, 29] and *Mycobacterium tuberculosis* FprA [30]. With the aim to partially fill the gap between the knowledge on the two types of FNR we searched the UniProt Knowledgebase (http://expasy.org/sprot) with the program BLAST for proteins homologous to human AdR, *Saccharomyces cerevisiae* ARH1 and *M. tuberculosis* FprA (SwissProt accession nos. **P22570**, **P48360** and **O05783**, respectively). A non-redundant set of retrieved sequences exhibiting similarity to the query sequences over their entire length were aligned using the program CLUSTALW [31]. Visual inspection of the conserved residues allowed us to define the consensus sequence of four highly conserved peptide segments (Table 1). As shown in Fig. 3, all these polypeptide motifs map in the active site of the AdR-like FNRs and make contacts with both FAD and NADP. Three of the motifs are involved in binding FAD. This binding site thus represents the most conserved feature in this FNR type. Motif 1 and 3 include the turns interacting with the pyrophosphate groups of FAD and NADP, respectively. More than 150 entries of the UniProt Knowledgebase contain all the four motifs of Table 1. Thus, they are likely to represent AdR-like enzymes, although several of them are annotated as unknown proteins or incorrectly identified (as, for example, the entry **A4TDE6**, which, although displaying more than 69% identity with FprA, is annotated as a disulfide oxidoreductase).

NADP(H) binding in different types of FNRs

In all FNR types, in order to allow for hydride transfer between the two redox-active moieties of the cosubstrate and the prosthetic group, NADP(H) binds to the enzyme in an extended conformation, with the 2'-P-AMP half accommodated within the NADP-binding domain and the NMN portion inserted in a cleft at the domain interface, where the nicotinamide ring can contact the FAD isoalloxazine. Various crystal forms of bovine AdR and *M. tuberculosis* FprA in complex with either $NADP^+$ or NADPH have been studied by X-ray crystallography [28, 30]. In all cases well-defined electron density maps have been obtained for the entire molecule of the bound ligand. In AdR-like enzymes, NADP(H) binds in a solvent accessible position, contacting several ordered water molecules. Minor conformational changes in the protein molecule have been observed as the result of NADP(H)-binding to AdR [28]. It can be concluded that in AdR-like FNRs the NADP-binding site is essentially preformed in the free enzyme and it is easily accessible by the ligand.

The interaction between NADP(H) and plant-type FNRs has been studied in detail from a structural point of view in both higher plant and *Anabaena* enzymes [4]. The NADP-binding site is much less solvent exposed in plant-type FNRs than in AdR-like enzymes. More interestingly, in the conformation of the free plant-type enzymes there is no room for binding the NMN moiety of the substrate in a catalytically competent conformation. Moreover, the X-ray analysis of the complex of spinach FNR with NADP yielded electron density only for the adenylate moiety of the ligand [17], while in *Anabaena* FNR, the NMN portion of $NADP^+$ was actually observed in crystal structures of the enzyme-substrate complex. Nevertheless, its conformation was not compatible with hydride transfer, *i. e.* the nicotinamide ring was far away from the isoalloxazine [23, 24]. The main structural feature disfavoring the occupation by the NMN moiety of its binding subsite is the conformation of the side-chain of the C-terminal Tyr that stacks on the *re*-face of the isoalloxazine, the site where the nicotinamide is expected to be during hydride transfer (Fig. 4). The C-terminal aromatic residue is highly conserved in plastidic-type FNRs, and it is also maintained in most members of the FNR superfamily [7, 14]. The current view of NADP(H) binding by plant-type FNRs during the catalysis, which takes into consideration results from the characterization of

several side-directed mutant forms, assumes that the NMN moiety of the bound substrate adopts a catalytically competent conformation only for a limited part of the catalytic cycle [20, 32, 33]. In other words, the NADP(H)-binding to plant-type FNR has been proposed to occur through a bipartite mechanism: the adenylate moiety has a leading role in enzyme-substrate interaction, and it remains firmly bound to the C-terminal domain in the NADP(H)-FNR complex, while the nicotinamide ring approaches the flavin ring only for a short fraction of the catalytic cycle, sufficient to provide the adequate hydride transfer rate [20, 32].

Catalysis of hydride transfer in FNRs

The catalytically competent conformation of NADP(H) bound to plastidic-type FNRs has been directly observed for the first time in the crystal structure of site-directed mutants of pea leaf FNR, where the C-terminal Tyr308 has been replaced with Ser or Trp (Fig. 4) [20]. This conformation, compatible with hydride transfer, has been confirmed by X-ray crystallographic studies on the corresponding mutant forms of cyanobacterial FNR [33], and by NMR studies on maize leaf FNR [34]. The residues most directly involved in the interaction with the nicotinamide ring of the cosubstrate have been shown to be Ser96, Cys272 and Glu312 (spinach leaf FNR numbering), and all of them are highly conserved in the plant-type FNRs. Glu312 makes an H-bond with the nicotinamide carboxamide [20, 35], while Ser96 and Cys272 interact with the C4 atom of the nicotinamide from opposing sides, possibly polarizing this position in order to favor hydride transfer [20, 36, 37]. Ser96 also interacts with the N5 atom of the FAD isoalloxazine [17]. The actual role of the triad formed by these residues (Ser, Cys, Glu) has been evaluated by protein engineering, showing that all of them contribute, although at different extents, to the k_{cat} of the plastidic-type enzymes [35-37].

A comparison of the active site of plant-type and AdR-like FNRs is shown in Fig. 5. Obviously, the environments of the bound nicotinamide ring differ substantially in the two groups of enzymes, as expected on the basis of their different stereospecificity [38, 39]. Indeed, whereas

hydride transfer involves the *re*-face of the flavin in all FNRs, it occurs at the A- or B-side of the nicotinamide ring in plant-type and GR-type FNRs, respectively [40]. A complete description of the stereochemistry of the interacting ring moieties in FNRs is given in Table 2. Notwithstanding large differences in active site organization, a common structural feature is present in all FNRs, namely, the side-chain carboxylate of a residue of the NADP-binding domain interacts with the nicotinamide carboxamide (Glu 211 in FprA; Glu312 and Glu306 in spinach leaf and pea leaf FNR, respectively; Fig. 5) [20, 30, 35]. An unusual feature of AdR-like FNRs is the lack of protein groups making direct contacts with the N5 and C4 positions of the isoalloxazine and nicotinamide rings, respectively [28-30]. In the crystal structure of the FprA-NADPH complex two ordered water molecules (water 1 and water 3 in Fig. 5) have been observed [30]. These water molecules lay at a position close to that occupied by the terminal groups of the side-chains of the active site Cys and Ser in plant-type FNRs. Thus, they are believed to have a role in favoring hydride transfer. Water 1 is likely highly reactive and it may initiate nicotinamide oxidation yielding 4-oxo-NADP (NADPO) when $NADP^+$ is incubated with *M. tuberculosis* FprA or bovine AdR [30, 41]. The ability of oxidize $NADP^+$ to NADPO seems a unique feature of AdR-like enzymes, which is absent in other types of FNR [41].

The highly divergent group of ONFR-like enzymes within the GR-type FNRs presents an active site highly dissimilar from that of AdR-like FNRs (Fig. 6) [13, 42-44]. Again, the only common feature is a carboxylate interacting with the nicotinamide ring (Glu159 in *Pseudomonas* BphA4), also in this case provided by the NAD-binding domain [13]. The side-chains of two residues, Glu159 and Lys53, conserved in ONFR-like enzymes and, more generally, in GR-related flavoproteins, interact with the nicotinamide C4 and the flavin N5 atoms, and they have been proposed to have a role in catalysis [13, 42]. However, site-directed mutagenesis studies on the corresponding residues (Glu313 and Lys176) of mouse AIF indicated their critical role in FAD binding and protein stability [44]. Lys176 replacement with Ala had a negligible effect on catalysis, while the Glu313Ala mutation mainly affected the K_m for NADPH [44].

Site-directed mutagenesis experiments targeting residues of GR-type FNRs putatively involved in hydride-transfer have also been performed on *M. tuberculosis* FprA. His57, whose side-chain interacts with both active-site water 1 and water 3, has been shown to markedly affect the ferredoxin-dependent reaction of the enzyme and to modulate the hydride transfer rate by controlling the precise positioning of the nicotinamide ring in the active site [45].

NADP/NAD selectivity

AdR-like FNRs discriminates against NADH essentially by means of a significantly higher values of K_m for this substrate in comparison to that for NADPH [46, 47]. On the other hand, plastidic-type FNRs display both lower values of K_m and higher values of k_{cat} for NADP(H) as compared to NAD(H) [18, 32, 48, 49]. As a result, plant-type FNRs are usually far more specific than AdR-like enzymes in cosubstrate preference. In principle, a much lower value of k_{cat} displayed by a NADPH-dependent enzyme when NADH is used as the electron donor can imply either an incorrect positioning of NADH in the active site or, alternatively, substrate binding through an induced fit mechanism that makes the enzyme active-site adopting its optimal conformation only when the 2'-phosphorylated cosubstrate is bound.

A major determinant of substrate specificity in all FNRs are the residues, some of which carrying a net positive charge, that directly interact with the 2'-phosphate of NADP(H). In AdR-like enzymes the negative charge of the 2'-phosphate is compensated by the side chain of Arg199 and Arg200 (FprA numbering) [28, 30]. Similarly, in plant-type FNRs, in addition to the hydroxyl groups of Ser234 and Tyr246, two positively-charged groups, provided by Arg235 and Lys244 (spinach leaf FNR numbering), make contacts with the 2'-phosphate [17, 23]. The role of these residues in substrate binding and catalysis has been studied in spinach and *Anabaena* FNRs by protein engineering [49-51]. Quite surprisingly, a second, very critical specificity determinant in plastidic-type FNR has been identified in the side chain of the C-terminal Tyr. Indeed, the Tyr308Ser mutation in pea FNR caused a drop in NADPH/NADH selectivity (measured as the ratio

of the k_{cat}/K_m for NADPH over that for NADH) from 36,000 to 77 [32]. Even more interestingly, the FNR-Y308S variant displayed a k_{cat} with NADH equal to that of the wild-type enzyme with NADPH [32]. This finding suggests that the side-chain of the C-terminal Tyr could have a role in the inducted-fit mechanism that prevents the enzyme from accepting the hydride ion from bound NADH with high efficiency.

Gomez-Moreno, Medina and coworkers have performed an exaustive analysis of the factors that govern the cosubstrate specificity in plant-type FNRs, and identified two additional peptide regions involved in the conformational changes that control NADP(H) binding, *i. e.* the loops of the NADP-binding domains involved in the interaction with the pyrophosphate moiety of the substrate [49, 52]. According to these authors, the NMN-binding subsite of plastidic-type FNRs has to undergo a general rearrangement and reshaping in order to nest the second half of the substrate, once its adenylate moiety is bound [52]. This reorganization should somehow be favored by the presence of the 2'-phosphate of NADP(H), although the underlying mechanism is still unknown.

Specific features of *Plasmodium falciparum* FNR

The phylum Apicomplexa comprises several species of protist parasites [53] that cause major human pathologies, including toxoplasmosis [54] and malaria [55]. These protozoa are characterized by specific organelles essential for parasite survival and virulence [53], including the apicoplast [56, 57], Most probably, the apicoplast represents the remnant of a secondary endosymbiotic event, in which the Apicomplexa ancestor engulfed an algal cell [56]. Consistently with its vegetal origin, the apicoplast possesses a typical plastidic metabolism [58], including isoprenoid and fatty acids biosyntheses, by using the methylerythritol 4-phosphate pathway [59], and type-II fatty acid synthase complex (FAS-II) [60], respectively. In the apicomplexan genomes several genes encoding homologs of plant proteins, harboring transit peptides for apicoplast targeting [61], have been identified, including Fd and FNR [2, 62].

T. gondii e *P. falciparum* FNRs have been cloned and characterized in detail [3, 22, 63-66]. Apicomplexan FNRs display highest sequence similarity to the root-type FNRs [62]. Whereas the kinetic properties of the *T. gondii* enzyme are very similar to those of other plastidic-type FNRs [65], the *P. falciparum* FNR displayed a significantly lower catalytic efficiency [22], as observed in bacterial-type FNRs [1]. The crystal structure of the enzyme has been solved in both the free and the 2'P-AMP bound forms [22]. The overall conformation of the polypeptide chain of the *P. falciparum* FNR is superposable to that of other plastidic-type FNRs (Fig. 7), although significant differences in important details were detected. The structural basis for the relatively low affinity of the plasmodial enzyme for NADP(H) is probably the lack of positively-charged groups stabilizing the 2'-phosphate of the bound substrate [22]. Another unexpected structural feature of the *P. falciparum* enzyme is a large conformational change that the site interacting with the 2'-P-AMP portion of NADP(H) undergoes when it is occupied [22]. Furthermore, a peculiar characteristic of the *P. falciparum* enzyme, unprecedented in other FNRs, is its susceptibility to undergo a NADP-dependent disulfide-linked dimerization process, resulting in enzyme inactivation [22]. The inactive homodimer produced in the presence of oxidizing agents, such as O_2, H_2O_2 or diamide, can be reconverted to the functional monomer by reducing agents such as DTT. The single disulfide bridge stabilizing the homodimer involves Cys99 from both enzyme protomers [22]. The reason for the low activity of the dimeric form of the enzyme (ca. 5% of that of the monomeric form) is most probably related to its quaternary structure, in which both electron transfer from reduced FAD, and $NADP^+$ release are hampered. Cys99 is highly, although not completely, conserved in plasmodial FNRs, and a Cys residue is present in the same sequence region also in the *T. gondii* homolog. The presence of the covalent dimer of apicoplast FNR *in vivo* has not been demonstrated yet. Nevertheless it could be suggested that inactivation by dimerization may represent part of a physiological process of FNR regulation in *Plasmodium*.

A possible anabolic role for the apicoplast FNR/Fd system has been demonstrated *in vitro*, through the reconstruction of a functional system comprising FNR, Fd, and LytB of *P. falciparum*

[3]. The latter enzyme catalyzes the last step of the mevalonate-independent 1-deoxy-D-xylulose-5-phosphate (DOXP) biosynthetic pathway of isoprenoid precursors. *P. falciparum* FNR/Fd system was found to be able to transfer to LytB the reducing equivalents needed to support its reductase activity [3]. Since isoprenoid biosynthesis is an essential pathway in *P. falciparum* and a known site of action of antimalarial drugs such as fosmidomycin [59], FNR represents a novel attractive target for the development of new antiplasmodial drugs [67].

Specific features of bacterial-type FNRs

Bacterial-type FNRs represent the most divergent group of plant-type FNRs [1]. Biochemically well-characterized members of this group of enzymes are from *Azotobacter vinelandii*, *Escherichia coli* and *Rhodobacter capsulatus*. Bacterial-type FNRs differ from plastidic-type FNRs by having additional physiological functions related to nitrogen fixation (in *Azotobacter* and *Rhodobacter*) and to the detoxification of reactive oxygen species, by using both Fd and flavodoxin as electron acceptors, and by displaying k_{cat} values two orders of magnitude lower than that of the plastidic-type counterparts [1]. Flavodoxin substitutes for Fd under conditions in which the [Fe-S] cluster of Fd cannot be assembled (e.g. under limiting iron availability) [5]. The use of flavodoxin as a redox partner is not restricted to bacterial-type FNRs, as it is an alternative substrate also for the plastidic-type FNRs of cyanobacteria and some algae [1]. One of the most peculiar structural features of the bacterial FNRs is the lack of the large FAD-binding domain loop that in plastidic-type enzymes interacts with the adenylate moiety of the prosthetic group (Fig. 2). As a result, in the former FNRs the FAD adopts a bent conformation with the adenine folded back and interacting with the isoalloxazine [1, 25-27]. Another striking difference between bacterial- and plastidic-type FNRs is that the stacking interaction between the *re*-face of the flavin and the aromatic side-chain of the C-terminal residue is lacking in both *A. vinelandii* [26] e *R. capsulatus* [27] enzymes .

Ferredoxin binding and electron-transfer

Electron transfer between FNR and Fd requires the transient formation of a protein-protein complex that puts their respective FAD and [Fe-S] prosthetic groups at a proper distance. The involvement of specific portions of the two proteins in the recognition process has been investigated using several different techniques, including chemical modification [68, 69], cross-linking [70], microcalorimetry [71, 72], protein engineering [5, 73-75], X-ray crystallography [19, 76-78], NMR [34, 79], and molecular interaction simulations [79]. A very large numbers of site-directed mutants of both *Anabaena* FNR and Fd have been characterized by transient absorption spectroscopy, leading to a quite complete picture of the respective interacting molecular surfaces and of the role of specific side chains in electron transfer [73]. Basically, the complex interface is formed by a hydrophobic core surrounded by charged residues, where basic side chains are mostly provided by the reductase and acidic ones by Fd. The dipole moment of the two protein molecules has probably a significant role in increasing the association rate and in favoring their mutual orientation [69]. The electron-transfer process is highly affected (up to four orders of magnitude) by non-conservative substitution at specific sites of the Fd surface: Phe65, Glu94, Ser47 (*Anabaena* numbering) [73]. The Fd-binding site of FNR was found to be much less sensitive to mutations, with the replacement of the most critical residues (Leu76, Lys75, Glu301, *Anabaena* numbering) leading to a decrease in the rate of electron transfer of up to 150 fold [73].

The crystal structure of four different Fd-FNR complexes has been solved by X-ray crystallography. Three of them involve plastidic-type proteins [19, 76, 78] and one AdR and adrenodoxin (Adx) [80]. In all cases, the iron-sulfur protein binds to a concave surface formed by both reductase domains. The resulting complexes have a compact, roughly spherical shape. The distance between the FAD and the [Fe-S] cluster is significantly shorter in the plastidic-type complexes (6-8 *vs* ca. 10 Å). The isoalloxazine ring of FAD is differently oriented with respect to the [Fe-S] cluster in the two types of complex. The pyridine ring of the flavin points towards the [Fe-S] cluster in the AdR-Adx complex, whereas the dimethylbenzene ring of the isoalloxazine faces the cluster of Fd in the plant-type complexes.

Adx binding to AdR induces a domain reorientation by several degrees in the latter protein molecule [80]. Small induced-fit conformational changes have been proposed to occur also in the formation of plant-type complexes [76, 78]. These backbone and side-chain movements have been proposed to favor the electron- and hydride-transfer steps of the catalytic cycle of plastidic-type FNRs [76, 78, 81]. It should be mentioned that Karplus [7] has proposed that, under physiological conditions, the photosynthetic FNR would always bind either $NADP^+$ or NADPH, while Fd would interact with the reductase in a collisional fashion, *i.e.* the protein-protein complex would be essentially unpopulated during the catalytic cycle. According to this view, any effect of Fd binding to the interaction between FNR and NADP(H) or to hydride transfer would be physiologically irrelevant.

Comparison of the crystal structure of the available plastidic-type FNR-Fd complexes has led to the unexpected observation that, although the structure of the corresponding proteins is highly conserved, the geometry of their interaction is not [76]. Indeed, whereas the interacting surfaces are the same in the three complexes, the proteins are differently oriented. In this respect it is interesting to mention the comprehensive survey of the intermolecular interacting surfaces in redox protein complexes carried out by Crowley and Carrondo [82]. Among the FNR-Fd complexes, the AdR-Adx couple is the one displaying the largest interface area with the highest number of H-bond and ionic interactions. The interfaces in plant-type FNR-Fd complexes are particularly poorly packed and display a low geometric fitting, in comparison to the non-redox protein complexes. In addition, while Coulombic attraction is predicted to be the major driving force determining the fast association rates of such complexes, it is not optimized in terms of precisely oriented intermolecular charge interactions. These structural properties are probably required to guarantee also a fast dissociation process and are known to be a general feature of the redox-protein complexes [82]. It seems reasonable to conclude that the heterogeneity in the mode of protein-protein interaction observed in the various plant-type FNR-Fd complexes probably reflects the absence of strict

geometric constrains in the interaction between the partners rather than actual species-specific differences.

Conclusions

FNR enzymology is representing for about half of a century a fertile and exciting area of research that had provided many clues on fundamental biochemical processes such as substrate recognition, protein-protein interaction, enzyme specificity and catalysis. Despite several important accomplishments, studies on FNRs are far from being concluded since a complete picture of their catalytic cycle is still lacking. FNRs endowed with novel properties from both bacteria and protozoa are being characterized and are expected to shed new light on the structure-function relationships of this important class of enzymes.

Acknowledgements

This work has been supported in part by a grant from the Ministry of University and Research of Italy and from Fondazione Cariplo, Milano, Italy.

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Figure legends

Figure 1. Structural/phylogenetic classification of ferredoxin-NAD(P)$^+$ reductases. Outline of the most relevant groups of FNRs, as determined by both sequence and three-dimensional structure comparison. Plant-type and GR-type FNRs represent two structurally/phylogenetically-unrelated protein families, making them a remarkable case of convergent evolution. On the other hand, further subdivisions within each family reflect increasing levels of evolutionary relationship.

Figure 2. Three-dimensional structure and domain organization of plant-type, bacterial-type, AdR-like and ONFR-like FNRs. A, *Plasmodium falciparum* FNR (PDB accession no. **2OK7**, chain A). **B**, *Escherichia coli* FNR (PDB accession no. **1FDR**). **C**, *Mycobacterium tuberculosis* FprA (PDB accession no. **1LQU**, chain A). **D**, *Pseudomonas* sp. Strain KKS102 BphA4 (PDB accession no. **1F3P**). Below each ribbon model, the contributions of the polypeptide chain to the different domains are reported. FAD-binding and NAD(P)-binding domains are shown in yellow and blue respectively. The C-terminal domain of BphA4 is shown in green. Bound FAD (A-D), 2'P-AMP (A), NADPH (C) and NADH (D) are represented as ball-and-stick models.

Figure 3. Highly conserved peptide regions in AdR-like FNRs. Ribbon model of the three-dimensional structure of FprA (1LQU, chain A) with FAD (right) and NADPH (left) represented as wireframes. The FAD-binding domain, the NADP-binding domain and the connecting β-sheet are colored in yellow, blue and magenta, respectively. The regions corresponding to the four sequence motifs highly conserved in AdR-like enzymes are highlighted in red. Motifs 1, 2 and 4 are located in the middle, upper and lower parts of the FAD-binding domain as depicted in the figure; motif 3 is located in the NADP-binding domain.

Figure 4. Induced-fit conformational change determined by NADP(H) binding to plant-type FNRs. Overlay of the three-dimensional structures of wild-type pea leaf FNR (PDB accession no. **1QG0**) (yellow ribbon) and NADP$^+$-bound pea leaf FNR-Y308S (PDB accession no. **1QFY**, chain

A). C-terminal Tyr308 and FAD belonging to wild-type FNR and $NADP^+$ bound to the mutant enzyme are shown as wireframes. Note how the nicotinamide ring of the bound co-substrate stacks on the isoalloxazine ring in the same position occupied by the Tyr308 side-chain in wild-type FNR.

Figure 5. Superposition of the active-site regions of AdR-like and plant-type FNRs. Stereo view of the groups involved in hydride-transfer between NAD(P)H and FAD in FprA (PDB accession no. **1LQU**, chain A) and leaf pea FNR-Y308S (PDB accession no. **1QFY**, chain A). FprA: NADPH, FAD, active-site acidic residues and ordered water 1 and water 3 are shown in red. FNR-Y308S: $NADP^+$, FAD, and relevant active-site residues are shown in green. Note how the terminal groups of the side chains of Cys266 (left) and Ser90 (right) of FNR-Y308S closely match the position of water 1 and 3, respectively, of FprA.

Figure 6. Superposition of the active-site regions of AdR-like and ONFR-like FNRs. Stereo view of the groups involved in hydride-transfer between NAD(P)H and FAD in FprA (PDB accession no. **1LQU**, chain A) and BphA4 (PDB accession no. **1D7Y**). FprA: NADPH, FAD, Glu214, Glu211, Asp161, His57, water 1 and water 3 are shown in red. BphA4: FAD, Glu159, Ser321, Ser52 and Lys53 are shown in blue. Note how the terminal groups of the side chains of Glu159 and Lys53 of BphA4 closely match the position of water 1 and 3, respectively, of FprA.

Figure 7. Comparison between the overall fold of photosynthetic and apicomplexan FNRs. Overlaid models of spinach leaf FNR (PDB accession no. **1FND**) and *P. falciparum* FNR (PDB accession no. **2OK7**, chain A). Spinach FNR backbone and ligands (FAD and 2'P-AMP) are shown as a green ribbon and dark green wireframes, respectively. *P. falciparum* FNR backbone and ligands (FAD and 2'P-AMP) are shown as an orange ribbon and red wireframes, respectively. Two alternative conformations of the 5'-phosphate group of the 2'P-AMP bound to spinach FNR are shown. Note that the adenylate moiety of the bound FAD adopts different conformations in the two FNRs, with the adenosine group rotated by ca. 180 degrees. The conformation of FAD in *P. falciparum* FNR is very similar to that observed in root-type FNRs.

Table 1. Conserved sequence motifs in AdR-like FNRs.

Motif	Consensus sequence (Prosite syntax)[1]	Sequence range in FprA	Domain	Function
1	[VI]-[VI]-G-X-G-P	8-13	FAD	FAD-pyrophosphate binding
2	G-L-X-R-X-G-X-A-P-D-H-X(3)-[KR]	47-61	FAD	FAD-isoalloxazine binding
3	G-X-G-N-V-X(2)-D-X(2)-R	154-164	NADP	NADP-pyrophosphate binding
4	G-W-X(3)-G-X(2)-G	358-366	FAD	FAD-ribityl binding

[1] http://www.expasy.ch/prosite [83].

Table 2. Complete configuration of the interacting NADP(H) and FAD in plant-type and GR-type FNRs [1]

FNR subgroup	NMN-flavin orientation	Nicotinamide		Flavin	
		Stereospecificity	Conformation	Stereospecificity	Conformation
Plant-type	exo	A	anti	re	anti
GR-type	endo	B	anti	re	syn

[1] The configuration of the nicotinamide/isoalloxazine couple is described according to the conventions adopted by Sem and Kasper [40]. The conformations of the nicotinamide and flavin moieties are both relative to the NMN ribose ring.

Structural and functional diversity of ferredoxin-NADP$^+$ reductases

Alessandro Aliverti*, Vittorio Pandini, Andrea Pennati[1], Matteo de Rosa, and Giuliana Zanetti

Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano, via Celoria 26, 20133 Milano, Italy.

[1]Current Address: Department of Chemistry, Georgia State University, Atlanta, GA 30302-4098, U.S.A.

Short title: Ferredoxin-NADP$^+$ reductases

*To which correspondence should be addressed at: Alessandro Aliverti, Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano, via Celoria 26, 20133 Milano, Italy, tel. +39 02 503 14897, fax +39 02 503 14895, e-mail alessandro.aliverti@unimi.it

Abstract

Although all ferredoxin-$NADP^+$ reductases (FNRs) catalyze the same reaction. *i. e.* the transfer of reducing equivalents between NADP(H) and ferredoxin, they belong to two unrelated families of proteins: the plant type and the glutathione reductase type of FNRs. Aim of this review is to provide a general classification scheme for these enzymes, to be used as a framework for the comparison of their properties. Furthermore, we report on some recent findings, which significantly increased the understanding of the structure-function relationships of FNRs, *i.e.* the ability of adrenodoxin reductase and its homologs to catalyze the oxidation of $NADP^+$ to its 4-oxo derivative, and the properties of plant-type FNRs from non-photosynthetic organisms. Plant-type FNRs from bacteria and Apicomplexan parasites provide examples of novel ways of FAD and NADP(H) binding. The recent characterization of an FNR from *Plasmodium falciparum* brings these enzymes into the field of drug design.

Keywords: Flavoprotein, FAD, NADP, photosynthesis, induced fit, electron transfer, Apicomplexa, *Plasmodium falciparum*, malaria

Abbreviations

AdR, adrenodoxin reductase; Adx, adrenodoxin; FNR, ferredoxin:$NADP^+$ oxidoreductase; Fd, ferredoxin; GR, glutathione reductase; NADPO, 4-oxo-NADP; NMN; nicotinamide mononucleotide; 2'-P-AMP, 2'-phospho-AMP.

Introduction

Ferredoxin:$NADP^+$ oxidoreductases (FNRs, EC 1.18.1.2) are FAD-containing flavoenzymes that catalyze the transfer of reducing equivalents between ferredoxins (Fds) and the $NADP^+$/NADPH couple according to the following equation [1]:

$$2Fd_{red} + NADP^+ = 2Fd_{ox} + NADPH \qquad \text{Eq. 1}$$

In several different organisms and tissues, FNRs participate in electron transfer chains involved in metabolic processes as diverse as photosynthesis, nitrogen fixation, isoprenoid biosynthesis, steroid metabolism, xenobiotic detoxification, oxidative-stress response, and iron-sulfur cluster biogenesis [1-5]. Eq. 1 represents the electron flow trough FNR as it occurs in the photosynthetic electron chain. However, the physiological direction of the reaction catalyzed by FNRs involved in the other pathways is opposite, *i. e.* toward the production of reduced Fd. On this basis, FNRs are sometimes classified as autotrophic (photosynthetic FNRs) and heterotrophic (all other FNRs) [6]. FNRs can be grouped into two phylogenetic/structural families that we here refer to as plant-type and glutathione reductase (GR)-type FNRs (Fig. 1). It should be noted that the term FNR is inconsistently used by different authors to denote either exclusively the plant-type FNRs or both enzyme families, thus bringing much confusion. A huge number of papers have been published on FNRs since the discovery of photosynthetic FNR and mitochondrial adrenodoxin reductase (AdR) in the sixties. Excellent reviews on plant-type FNRs appeared in recent years, and the reader is referred to them for an introduction to the field [1, 4, 5, 7]. To the best of our knowledge, no reviews on the GR-type FNRs have been published so far. The only article about the general properties of AdR appeared more than 15 years ago [8]. The scope of the present review is to cover aspects not adequately considered in previous work, with a special focus on two topics. First, we will provide a comparison between plant-type and GR-type FNRs. Second, we will summarize the most recent findings on these two highly important enzyme families.

Structural and phylogenetic classification of FNRs

As anticipated in the Introduction, enzymes that catalyze the reaction described in Eq. 1, and thus referred to as FNRs, belong to two phylogenetically and structurally unrelated protein families. The sequences of several hundred proteins annotated as FNR have been deposited in the SwissProt and GenBank NCBI databases. More than 50 entries dealing with the crystal structures of FNRs are also currently available in the Protein Data Bank. By comparison of their three-dimensional structures and sequences the two FNR families have been further subdivided as outlined in Fig. 1. Thus plant-type FNRs comprise the plastidic and bacterial enzymes, whereas the AdR-like flavoproteins and, for the reasons detailed below, the ONFRs belong to the GR-type FNRs. FNRs of both families possess a two-domain organization with the active site located at the interface between the FAD- and the NADP-binding domains (Fig. 2). Dym and Eisenberg have identified 4 general folds for FAD binding in 32 families of FAD-containing proteins [9]. The FAD-binding domain of the GR-type and the plant-type FNRs adopt two of such general folds.

The above subdivision of FNRs in two evolutionary families is in agreement with both CATH (http://www.cathdb.info) and SCOP (http://scop.berkeley.edu) classifications of protein domains [10, 11]. The FAD-binding domain of plant-type FNRs is based on an antiparallel β-barrel with a greek-key topology (number of strand, $n = 6$, and shear number, $S = 10$) [12]. The corresponding domain of AdR-like FNRs adopts a three-layer β/β/α fold, with a central parallel five-membered β-sheet of 32145 topology. It should be noted that, while in plant-type FNRs the FAD-binding domain is formed by the N-terminal portion of the polypeptide chain, in GR-type enzymes two discontinuous segments of the polypeptide form the FAD-binding domain (Fig. 2). The NADP-binding domain of both FNR families is a three-layer sandwich α/β/α, with a Rossmann-like topology and a parallel 5-membered β-sheet. However, these domains differ in several details in the two families of FNRs, particularly in the precise mode in which NADP(H) is bound.

It is worth to mention that a family of flavoproteins exists, which, although NAD-dependent, are clearly structurally and functionally related to the AdR-like FNRs. This family includes the bacterial oxygenase-coupled NADH-ferredoxin reductases (ONFRs), of which the *Pseudomonas* ferredoxin reductase component of biphenyl dioxygenase (BphA4) is the best characterized member [13]. ONFR-like enzymes (EC 1.18.1.3), though not formally FNRs, will be shortly dealt with in this review, since their properties help in understanding the structure-function relationships of AdR-like proteins. ONFRs-like enzymes mainly differ from AdR-like FNRs in having both FAD- and NAD-domain sharing the same organization (a three-layer β/β/α fold related to the FAD-binding domain of AdR) and in possessing a third C-terminal domain involved in protein homodimerization (Fig. 2).

Finally, it is interesting to note that both families of FNRs are part of wider protein superfamilies that include non-FNR members. The "FNR superfamily" consists of proteins that share the two-domain unit of plant-type FNR and possess a variety of additional domains [7, 14, 15]. It comprises nitrate reductase, phthalate dioxygenase reductase, sulfite reductase and the dual flavin oxidoreductases such as cytochrome P450 reductase, NO synthase and methionine synthase reductase. Similarly, AdR-like and ONFR-like enzymes belong to the "two dinucleotide binding domains" flavoprotein superfamily (tDBDF) [16], which includes the Baeyer-Villiger monooxygenase, glutamate synthase β subunit, dehydropyridine dehydrogenase, thioredoxin reductase, and glutathione reductase.

Plant-type FNRs have been studied extensively, with the functional and structural characterization of enzymes from several eukaryotic and prokaryotic organisms. In particular, high resolution three-dimensional structures have been solved for the FNRs from spinach (*Spinacia oleracea*) leaf [17], corn (*Zea mays*) leaf and root [18, 19], pea (*Pisum sativum*) leaf [20], paprika (*Capsicum annuum*) leaf [21], the protozoon *Plasmodium falciparum* [22], the cyanobacteria *Anabaena* [23, 24] and *Synecochococcus* (PDB accession no. **2B5O**), and the bacteria *Escherichia coli* [25], *Azotobacter vinelandii* [26] and *Rhodobacter capsulatus* [27]. The 3D structure and the

amino acid sequences of these proteins have been compared in detail leading to the definition of the consensus of six sequence motifs highly conserved in plant-type FNRs [1] and to the identification of the groups in which these enzymes can be further subdivided (Fig. 1), each characterized by specific structural features [1, 7].

Conserved structural features in AdR-like FNRs

As mentioned before, AdR-like FNRs have been studied to a lower extent than the plant-type ones. For instance, three-dimensional structures have been obtained only for bovine AdR [28, 29] and *Mycobacterium tuberculosis* FprA [30]. With the aim to partially fill the gap between the knowledge on the two types of FNR we searched the UniProt Knowledgebase (http://expasy.org/sprot) with the program BLAST for proteins homologous to human AdR, *Saccharomyces cerevisiae* ARH1 and *M. tuberculosis* FprA (SwissProt accession nos. **P22570**, **P48360** and **O05783**, respectively). A non-redundant set of retrieved sequences exhibiting similarity to the query sequences over their entire length were aligned using the program CLUSTALW [31]. Visual inspection of the conserved residues allowed us to define the consensus sequence of four highly conserved peptide segments (Table 1). As shown in Fig. 3, all these polypeptide motifs map in the active site of the AdR-like FNRs and make contacts with both FAD and NADP. Three of the motifs are involved in binding FAD. This binding site thus represents the most conserved feature in this FNR type. Motif 1 and 3 include the turns interacting with the pyrophosphate groups of FAD and NADP, respectively. More than 150 entries of the UniProt Knowledgebase contain all the four motifs of Table 1. Thus, they are likely to represent AdR-like enzymes, although several of them are annotated as unknown proteins or incorrectly identified (as, for example, the entry **A4TDE6**, which, although displaying more than 69% identity with FprA, is annotated as a disulfide oxidoreductase).

NADP(H) binding in different types of FNRs

In all FNR types, in order to allow for hydride transfer between the two redox-active moieties of the cosubstrate and the prosthetic group, NADP(H) binds to the enzyme in an extended conformation, with the 2'-P-AMP half accommodated within the NADP-binding domain and the NMN portion inserted in a cleft at the domain interface, where the nicotinamide ring can contact the FAD isoalloxazine. Various crystal forms of bovine AdR and *M. tuberculosis* FprA in complex with either $NADP^+$ or NADPH have been studied by X-ray crystallography [28, 30]. In all cases well-defined electron density maps have been obtained for the entire molecule of the bound ligand. In AdR-like enzymes, NADP(H) binds in a solvent accessible position, contacting several ordered water molecules. Minor conformational changes in the protein molecule have been observed as the result of NADP(H)-binding to AdR [28]. It can be concluded that in AdR-like FNRs the NADP-binding site is essentially preformed in the free enzyme and it is easily accessible by the ligand.

The interaction between NADP(H) and plant-type FNRs has been studied in detail from a structural point of view in both higher plant and *Anabaena* enzymes [4]. The NADP-binding site is much less solvent exposed in plant-type FNRs than in AdR-like enzymes. More interestingly, in the conformation of the free plant-type enzymes there is no room for binding the NMN moiety of the substrate in a catalytically competent conformation. Moreover, the X-ray analysis of the complex of spinach FNR with NADP yielded electron density only for the adenylate moiety of the ligand [17], while in *Anabaena* FNR, the NMN portion of $NADP^+$ was actually observed in crystal structures of the enzyme-substrate complex. Nevertheless, its conformation was not compatible with hydride transfer, *i. e.* the nicotinamide ring was far away from the isoalloxazine [23, 24]. The main structural feature disfavoring the occupation by the NMN moiety of its binding subsite is the conformation of the side-chain of the C-terminal Tyr that stacks on the *re*-face of the isoalloxazine, the site where the nicotinamide is expected to be during hydride transfer (Fig. 4). The C-terminal aromatic residue is highly conserved in plastidic-type FNRs, and it is also maintained in most members of the FNR superfamily [7, 14]. The current view of NADP(H) binding by plant-type FNRs during the catalysis, which takes into consideration results from the characterization of

several side-directed mutant forms, assumes that the NMN moiety of the bound substrate adopts a catalytically competent conformation only for a limited part of the catalytic cycle [20, 32, 33]. In other words, the NADP(H)-binding to plant-type FNR has been proposed to occur through a bipartite mechanism: the adenylate moiety has a leading role in enzyme-substrate interaction, and it remains firmly bound to the C-terminal domain in the NADP(H)-FNR complex, while the nicotinamide ring approaches the flavin ring only for a short fraction of the catalytic cycle, sufficient to provide the adequate hydride transfer rate [20, 32].

Catalysis of hydride transfer in FNRs

The catalytically competent conformation of NADP(H) bound to plastidic-type FNRs has been directly observed for the first time in the crystal structure of site-directed mutants of pea leaf FNR, where the C-terminal Tyr308 has been replaced with Ser or Trp (Fig. 4) [20]. This conformation, compatible with hydride transfer, has been confirmed by X-ray crystallographic studies on the corresponding mutant forms of cyanobacterial FNR [33], and by NMR studies on maize leaf FNR [34]. The residues most directly involved in the interaction with the nicotinamide ring of the cosubstrate have been shown to be Ser96, Cys272 and Glu312 (spinach leaf FNR numbering), and all of them are highly conserved in the plant-type FNRs. Glu312 makes an H-bond with the nicotinamide carboxamide [20, 35], while Ser96 and Cys272 interact with the C4 atom of the nicotinamide from opposing sides, possibly polarizing this position in order to favor hydride transfer [20, 36, 37]. Ser96 also interacts with the N5 atom of the FAD isoalloxazine [17]. The actual role of the triad formed by these residues (Ser, Cys, Glu) has been evaluated by protein engineering, showing that all of them contribute, although at different extents, to the k_{cat} of the plastidic-type enzymes [35-37].

A comparison of the active site of plant-type and AdR-like FNRs is shown in Fig. 5. Obviously, the environments of the bound nicotinamide ring differ substantially in the two groups of enzymes, as expected on the basis of their different stereospecificity [38, 39]. Indeed, whereas

hydride transfer involves the *re*-face of the flavin in all FNRs, it occurs at the A- or B-side of the nicotinamide ring in plant-type and GR-type FNRs, respectively [40]. A complete description of the stereochemistry of the interacting ring moieties in FNRs is given in Table 2. Notwithstanding large differences in active site organization, a common structural feature is present in all FNRs, namely, the side-chain carboxylate of a residue of the NADP-binding domain interacts with the nicotinamide carboxamide (Glu 211 in FprA; Glu312 and Glu306 in spinach leaf and pea leaf FNR, respectively; Fig. 5) [20, 30, 35]. An unusual feature of AdR-like FNRs is the lack of protein groups making direct contacts with the N5 and C4 positions of the isoalloxazine and nicotinamide rings, respectively [28-30]. In the crystal structure of the FprA-NADPH complex two ordered water molecules (water 1 and water 3 in Fig. 5) have been observed [30]. These water molecules lay at a position close to that occupied by the terminal groups of the side-chains of the active site Cys and Ser in plant-type FNRs. Thus, they are believed to have a role in favoring hydride transfer. Water 1 is likely highly reactive and it may initiate nicotinamide oxidation yielding 4-oxo-NADP (NADPO) when $NADP^+$ is incubated with *M. tuberculosis* FprA or bovine AdR [30, 41]. The ability of oxidize $NADP^+$ to NADPO seems a unique feature of AdR-like enzymes, which is absent in other types of FNR [41].

The highly divergent group of ONFR-like enzymes within the GR-type FNRs presents an active site highly dissimilar from that of AdR-like FNRs (Fig. 6) [13, 42-44]. Again, the only common feature is a carboxylate interacting with the nicotinamide ring (Glu159 in *Pseudomonas* BphA4), also in this case provided by the NAD-binding domain [13]. The side-chains of two residues, Glu159 and Lys53, conserved in ONFR-like enzymes and, more generally, in GR-related flavoproteins, interact with the nicotinamide C4 and the flavin N5 atoms, and they have been proposed to have a role in catalysis [13, 42]. However, site-directed mutagenesis studies on the corresponding residues (Glu313 and Lys176) of mouse AIF indicated their critical role in FAD binding and protein stability [44]. Lys176 replacement with Ala had a negligible effect on catalysis, while the Glu313Ala mutation mainly affected the K_m for NADPH [44].

Site-directed mutagenesis experiments targeting residues of GR-type FNRs putatively involved in hydride-transfer have also been performed on *M. tuberculosis* FprA. His57, whose side-chain interacts with both active-site water 1 and water 3, has been shown to markedly affect the ferredoxin-dependent reaction of the enzyme and to modulate the hydride transfer rate by controlling the precise positioning of the nicotinamide ring in the active site [45].

NADP/NAD selectivity

AdR-like FNRs discriminates against NADH essentially by means of a significantly higher values of K_m for this substrate in comparison to that for NADPH [46, 47]. On the other hand, plastidic-type FNRs display both lower values of K_m and higher values of k_{cat} for NADP(H) as compared to NAD(H) [18, 32, 48, 49]. As a result, plant-type FNRs are usually far more specific than AdR-like enzymes in cosubstrate preference. In principle, a much lower value of k_{cat} displayed by a NADPH-dependent enzyme when NADH is used as the electron donor can imply either an incorrect positioning of NADH in the active site or, alternatively, substrate binding through an induced fit mechanism that makes the enzyme active-site adopting its optimal conformation only when the 2'-phosphorylated cosubstrate is bound.

A major determinant of substrate specificity in all FNRs are the residues, some of which carrying a net positive charge, that directly interact with the 2'-phosphate of NADP(H). In AdR-like enzymes the negative charge of the 2'-phosphate is compensated by the side chain of Arg199 and Arg200 (FprA numbering) [28, 30]. Similarly, in plant-type FNRs, in addition to the hydroxyl groups of Ser234 and Tyr246, two positively-charged groups, provided by Arg235 and Lys244 (spinach leaf FNR numbering), make contacts with the 2'-phosphate [17, 23]. The role of these residues in substrate binding and catalysis has been studied in spinach and *Anabaena* FNRs by protein engineering [49-51]. Quite surprisingly, a second, very critical specificity determinant in plastidic-type FNR has been identified in the side chain of the C-terminal Tyr. Indeed, the Tyr308Ser mutation in pea FNR caused a drop in NADPH/NADH selectivity (measured as the ratio

of the k_{cat}/K_m for NADPH over that for NADH) from 36,000 to 77 [32]. Even more interestingly, the FNR-Y308S variant displayed a k_{cat} with NADH equal to that of the wild-type enzyme with NADPH [32]. This finding suggests that the side-chain of the C-terminal Tyr could have a role in the inducted-fit mechanism that prevents the enzyme from accepting the hydride ion from bound NADH with high efficiency.

Gomez-Moreno, Medina and coworkers have performed an exaustive analysis of the factors that govern the cosubstrate specificity in plant-type FNRs, and identified two additional peptide regions involved in the conformational changes that control NADP(H) binding, *i. e.* the loops of the NADP-binding domains involved in the interaction with the pyrophosphate moiety of the substrate [49, 52]. According to these authors, the NMN-binding subsite of plastidic-type FNRs has to undergo a general rearrangement and reshaping in order to nest the second half of the substrate, once its adenylate moiety is bound [52]. This reorganization should somehow be favored by the presence of the 2'-phosphate of NADP(H), although the underlying mechanism is still unknown.

Specific features of *Plasmodium falciparum* FNR

The phylum Apicomplexa comprises several species of protist parasites [53] that cause major human pathologies, including toxoplasmosis [54] and malaria [55]. These protozoa are characterized by specific organelles essential for parasite survival and virulence [53], including the apicoplast [56, 57], Most probably, the apicoplast represents the remnant of a secondary endosymbiotic event, in which the Apicomplexa ancestor engulfed an algal cell [56]. Consistently with its vegetal origin, the apicoplast possesses a typical plastidic metabolism [58], including isoprenoid and fatty acids biosyntheses, by using the methylerythritol 4-phosphate pathway [59], and type-II fatty acid synthase complex (FAS-II) [60], respectively. In the apicomplexan genomes several genes encoding homologs of plant proteins, harboring transit peptides for apicoplast targeting [61], have been identified, including Fd and FNR [2, 62].

T. gondii e *P. falciparum* FNRs have been cloned and characterized in detail [3, 22, 63-66]. Apicomplexan FNRs display highest sequence similarity to the root-type FNRs [62]. Whereas the kinetic properties of the *T. gondii* enzyme are very similar to those of other plastidic-type FNRs [65], the *P. falciparum* FNR displayed a significantly lower catalytic efficiency [22], as observed in bacterial-type FNRs [1]. The crystal structure of the enzyme has been solved in both the free and the 2'P-AMP bound forms [22]. The overall conformation of the polypeptide chain of the *P. falciparum* FNR is superposable to that of other plastidic-type FNRs (Fig. 7), although significant differences in important details were detected. The structural basis for the relatively low affinity of the plasmodial enzyme for NADP(H) is probably the lack of positively-charged groups stabilizing the 2'-phosphate of the bound substrate [22]. Another unexpected structural feature of the *P. falciparum* enzyme is a large conformational change that the site interacting with the 2'-P-AMP portion of NADP(H) undergoes when it is occupied [22]. Furthermore, a peculiar characteristic of the *P. falciparum* enzyme, unprecedented in other FNRs, is its susceptibility to undergo a NADP-dependent disulfide-linked dimerization process, resulting in enzyme inactivation [22]. The inactive homodimer produced in the presence of oxidizing agents, such as O_2, H_2O_2 or diamide, can be reconverted to the functional monomer by reducing agents such as DTT. The single disulfide bridge stabilizing the homodimer involves Cys99 from both enzyme protomers [22]. The reason for the low activity of the dimeric form of the enzyme (ca. 5% of that of the monomeric form) is most probably related to its quaternary structure, in which both electron transfer from reduced FAD, and $NADP^+$ release are hampered. Cys99 is highly, although not completely, conserved in plasmodial FNRs, and a Cys residue is present in the same sequence region also in the *T. gondii* homolog. The presence of the covalent dimer of apicoplast FNR *in vivo* has not been demonstrated yet. Nevertheless it could be suggested that inactivation by dimerization may represent part of a physiological process of FNR regulation in *Plasmodium*.

A possible anabolic role for the apicoplast FNR/Fd system has been demonstrated *in vitro*, through the reconstruction of a functional system comprising FNR, Fd, and LytB of *P. falciparum*

[3]. The latter enzyme catalyzes the last step of the mevalonate-independent 1-deoxy-D-xylulose-5-phosphate (DOXP) biosynthetic pathway of isoprenoid precursors. *P. falciparum* FNR/Fd system was found to be able to transfer to LytB the reducing equivalents needed to support its reductase activity [3]. Since isoprenoid biosynthesis is an essential pathway in *P. falciparum* and a known site of action of antimalarial drugs such as fosmidomycin [59], FNR represents a novel attractive target for the development of new antiplasmodial drugs [67].

Specific features of bacterial-type FNRs

Bacterial-type FNRs represent the most divergent group of plant-type FNRs [1]. Biochemically well-characterized members of this group of enzymes are from *Azotobacter vinelandii*, *Escherichia coli* and *Rhodobacter capsulatus*. Bacterial-type FNRs differ from plastidic-type FNRs by having additional physiological functions related to nitrogen fixation (in *Azotobacter* and *Rhodobacter*) and to the detoxification of reactive oxygen species, by using both Fd and flavodoxin as electron acceptors, and by displaying k_{cat} values two orders of magnitude lower than that of the plastidic-type counterparts [1]. Flavodoxin substitutes for Fd under conditions in which the [Fe-S] cluster of Fd cannot be assembled (e.g. under limiting iron availability) [5]. The use of flavodoxin as a redox partner is not restricted to bacterial-type FNRs, as it is an alternative substrate also for the plastidic-type FNRs of cyanobacteria and some algae [1]. One of the most peculiar structural features of the bacterial FNRs is the lack of the large FAD-binding domain loop that in plastidic-type enzymes interacts with the adenylate moiety of the prosthetic group (Fig. 2). As a result, in the former FNRs the FAD adopts a bent conformation with the adenine folded back and interacting with the isoalloxazine [1, 25-27]. Another striking difference between bacterial- and plastidic-type FNRs is that the stacking interaction between the *re*-face of the flavin and the aromatic side-chain of the C-terminal residue is lacking in both *A. vinelandii* [26] e *R. capsulatus* [27] enzymes .

Ferredoxin binding and electron-transfer

Electron transfer between FNR and Fd requires the transient formation of a protein-protein complex that puts their respective FAD and [Fe-S] prosthetic groups at a proper distance. The involvement of specific portions of the two proteins in the recognition process has been investigated using several different techniques, including chemical modification [68, 69], cross-linking [70], microcalorimetry [71, 72], protein engineering [5, 73-75], X-ray crystallography [19, 76-78], NMR [34, 79], and molecular interaction simulations [79]. A very large numbers of site-directed mutants of both *Anabaena* FNR and Fd have been characterized by transient absorption spectroscopy, leading to a quite complete picture of the respective interacting molecular surfaces and of the role of specific side chains in electron transfer [73]. Basically, the complex interface is formed by a hydrophobic core surrounded by charged residues, where basic side chains are mostly provided by the reductase and acidic ones by Fd. The dipole moment of the two protein molecules has probably a significant role in increasing the association rate and in favoring their mutual orientation [69]. The electron-transfer process is highly affected (up to four orders of magnitude) by non-conservative substitution at specific sites of the Fd surface: Phe65, Glu94, Ser47 (*Anabaena* numbering) [73]. The Fd-binding site of FNR was found to be much less sensitive to mutations, with the replacement of the most critical residues (Leu76, Lys75, Glu301, *Anabaena* numbering) leading to a decrease in the rate of electron transfer of up to 150 fold [73].

The crystal structure of four different Fd-FNR complexes has been solved by X-ray crystallography. Three of them involve plastidic-type proteins [19, 76, 78] and one AdR and adrenodoxin (Adx) [80]. In all cases, the iron-sulfur protein binds to a concave surface formed by both reductase domains. The resulting complexes have a compact, roughly spherical shape. The distance between the FAD and the [Fe-S] cluster is significantly shorter in the plastidic-type complexes (6-8 *vs* ca. 10 Å). The isoalloxazine ring of FAD is differently oriented with respect to the [Fe-S] cluster in the two types of complex. The pyridine ring of the flavin points towards the [Fe-S] cluster in the AdR-Adx complex, whereas the dimethylbenzene ring of the isoalloxazine faces the cluster of Fd in the plant-type complexes.

Adx binding to AdR induces a domain reorientation by several degrees in the latter protein molecule [80]. Small induced-fit conformational changes have been proposed to occur also in the formation of plant-type complexes [76, 78]. These backbone and side-chain movements have been proposed to favor the electron- and hydride-transfer steps of the catalytic cycle of plastidic-type FNRs [76, 78, 81]. It should be mentioned that Karplus [7] has proposed that, under physiological conditions, the photosynthetic FNR would always bind either $NADP^+$ or NADPH, while Fd would interact with the reductase in a collisional fashion, *i.e.* the protein-protein complex would be essentially unpopulated during the catalytic cycle. According to this view, any effect of Fd binding to the interaction between FNR and NADP(H) or to hydride transfer would be physiologically irrelevant.

Comparison of the crystal structure of the available plastidic-type FNR-Fd complexes has led to the unexpected observation that, although the structure of the corresponding proteins is highly conserved, the geometry of their interaction is not [76]. Indeed, whereas the interacting surfaces are the same in the three complexes, the proteins are differently oriented. In this respect it is interesting to mention the comprehensive survey of the intermolecular interacting surfaces in redox protein complexes carried out by Crowley and Carrondo [82]. Among the FNR-Fd complexes, the AdR-Adx couple is the one displaying the largest interface area with the highest number of H-bond and ionic interactions. The interfaces in plant-type FNR-Fd complexes are particularly poorly packed and display a low geometric fitting, in comparison to the non-redox protein complexes. In addition, while Coulombic attraction is predicted to be the major driving force determining the fast association rates of such complexes, it is not optimized in terms of precisely oriented intermolecular charge interactions. These structural properties are probably required to guarantee also a fast dissociation process and are known to be a general feature of the redox-protein complexes [82]. It seems reasonable to conclude that the heterogeneity in the mode of protein-protein interaction observed in the various plant-type FNR-Fd complexes probably reflects the absence of strict

geometric constrains in the interaction between the partners rather than actual species-specific differences.

Conclusions

FNR enzymology is representing for about half of a century a fertile and exciting area of research that had provided many clues on fundamental biochemical processes such as substrate recognition, protein-protein interaction, enzyme specificity and catalysis. Despite several important accomplishments, studies on FNRs are far from being concluded since a complete picture of their catalytic cycle is still lacking. FNRs endowed with novel properties from both bacteria and protozoa are being characterized and are expected to shed new light on the structure-function relationships of this important class of enzymes.

Acknowledgements

This work has been supported in part by a grant from the Ministry of University and Research of Italy and from Fondazione Cariplo, Milano, Italy.

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Figure legends

Figure 1. Structural/phylogenetic classification of ferredoxin-NAD(P)$^+$ reductases. Outline of the most relevant groups of FNRs, as determined by both sequence and three-dimensional structure comparison. Plant-type and GR-type FNRs represent two structurally/phylogenetically-unrelated protein families, making them a remarkable case of convergent evolution. On the other hand, further subdivisions within each family reflect increasing levels of evolutionary relationship.

Figure 2. Three-dimensional structure and domain organization of plant-type, bacterial-type, AdR-like and ONFR-like FNRs. A, *Plasmodium falciparum* FNR (PDB accession no. **2OK7**, chain A). **B**, *Escherichia coli* FNR (PDB accession no. **1FDR**). **C**, *Mycobacterium tuberculosis* FprA (PDB accession no. **1LQU**, chain A). **D**, *Pseudomonas* sp. Strain KKS102 BphA4 (PDB accession no. **1F3P**). Below each ribbon model, the contributions of the polypeptide chain to the different domains are reported. FAD-binding and NAD(P)-binding domains are shown in yellow and blue respectively. The C-terminal domain of BphA4 is shown in green. Bound FAD (A-D), 2'P-AMP (A), NADPH (C) and NADH (D) are represented as ball-and-stick models.

Figure 3. Highly conserved peptide regions in AdR-like FNRs. Ribbon model of the three-dimensional structure of FprA (1LQU, chain A) with FAD (right) and NADPH (left) represented as wireframes. The FAD-binding domain, the NADP-binding domain and the connecting β-sheet are colored in yellow, blue and magenta, respectively. The regions corresponding to the four sequence motifs highly conserved in AdR-like enzymes are highlighted in red. Motifs 1, 2 and 4 are located in the middle, upper and lower parts of the FAD-binding domain as depicted in the figure; motif 3 is located in the NADP-binding domain.

Figure 4. Induced-fit conformational change determined by NADP(H) binding to plant-type FNRs. Overlay of the three-dimensional structures of wild-type pea leaf FNR (PDB accession no. **1QG0**) (yellow ribbon) and NADP$^+$-bound pea leaf FNR-Y308S (PDB accession no. **1QFY**, chain

A). C-terminal Tyr308 and FAD belonging to wild-type FNR and $NADP^+$ bound to the mutant enzyme are shown as wireframes. Note how the nicotinamide ring of the bound co-substrate stacks on the isoalloxazine ring in the same position occupied by the Tyr308 side-chain in wild-type FNR.

Figure 5. Superposition of the active-site regions of AdR-like and plant-type FNRs. Stereo view of the groups involved in hydride-transfer between NAD(P)H and FAD in FprA (PDB accession no. **1LQU**, chain A) and leaf pea FNR-Y308S (PDB accession no. **1QFY**, chain A). FprA: NADPH, FAD, active-site acidic residues and ordered water 1 and water 3 are shown in red. FNR-Y308S: $NADP^+$, FAD, and relevant active-site residues are shown in green. Note how the terminal groups of the side chains of Cys266 (left) and Ser90 (right) of FNR-Y308S closely match the position of water 1 and 3, respectively, of FprA.

Figure 6. Superposition of the active-site regions of AdR-like and ONFR-like FNRs. Stereo view of the groups involved in hydride-transfer between NAD(P)H and FAD in FprA (PDB accession no. **1LQU**, chain A) and BphA4 (PDB accession no. **1D7Y**). FprA: NADPH, FAD, Glu214, Glu211, Asp161, His57, water 1 and water 3 are shown in red. BphA4: FAD, Glu159, Ser321, Ser52 and Lys53 are shown in blue. Note how the terminal groups of the side chains of Glu159 and Lys53 of BphA4 closely match the position of water 1 and 3, respectively, of FprA.

Figure 7. Comparison between the overall fold of photosynthetic and apicomplexan FNRs. Overlaid models of spinach leaf FNR (PDB accession no. **1FND**) and *P. falciparum* FNR (PDB accession no. **2OK7**, chain A). Spinach FNR backbone and ligands (FAD and 2'P-AMP) are shown as a green ribbon and dark green wireframes, respectively. *P. falciparum* FNR backbone and ligands (FAD and 2'P-AMP) are shown as an orange ribbon and red wireframes, respectively. Two alternative conformations of the 5'-phosphate group of the 2'P-AMP bound to spinach FNR are shown. Note that the adenylate moiety of the bound FAD adopts different conformations in the two FNRs, with the adenosine group rotated by ca. 180 degrees. The conformation of FAD in *P. falciparum* FNR is very similar to that observed in root-type FNRs.

Table 1. Conserved sequence motifs in AdR-like FNRs.

Motif	Consensus sequence (Prosite syntax)[1]	Sequence range in FprA	Domain	Function
1	[VI]-[VI]-G-X-G-P	8-13	FAD	FAD-pyrophosphate binding
2	G-L-X-R-X-G-X-A-P-D-H-X(3)-[KR]	47-61	FAD	FAD-isoalloxazine binding
3	G-X-G-N-V-X(2)-D-X(2)-R	154-164	NADP	NADP-pyrophosphate binding
4	G-W-X(3)-G-X(2)-G	358-366	FAD	FAD-ribityl binding

[1] http://www.expasy.ch/prosite [83].

Table 2. Complete configuration of the interacting NADP(H) and FAD in plant-type and GR-type FNRs [1]

FNR subgroup	NMN-flavin orientation	Nicotinamide		Flavin	
		Stereospecificity	Conformation	Stereospecificity	Conformation
Plant-type	exo	A	anti	re	anti
GR-type	endo	B	anti	re	syn

[1] The configuration of the nicotinamide/isoalloxazine couple is described according to the conventions adopted by Sem and Kasper [40]. The conformations of the nicotinamide and flavin moieties are both relative to the NMN ribose ring.

Fig_1
Click here to download high resolution image

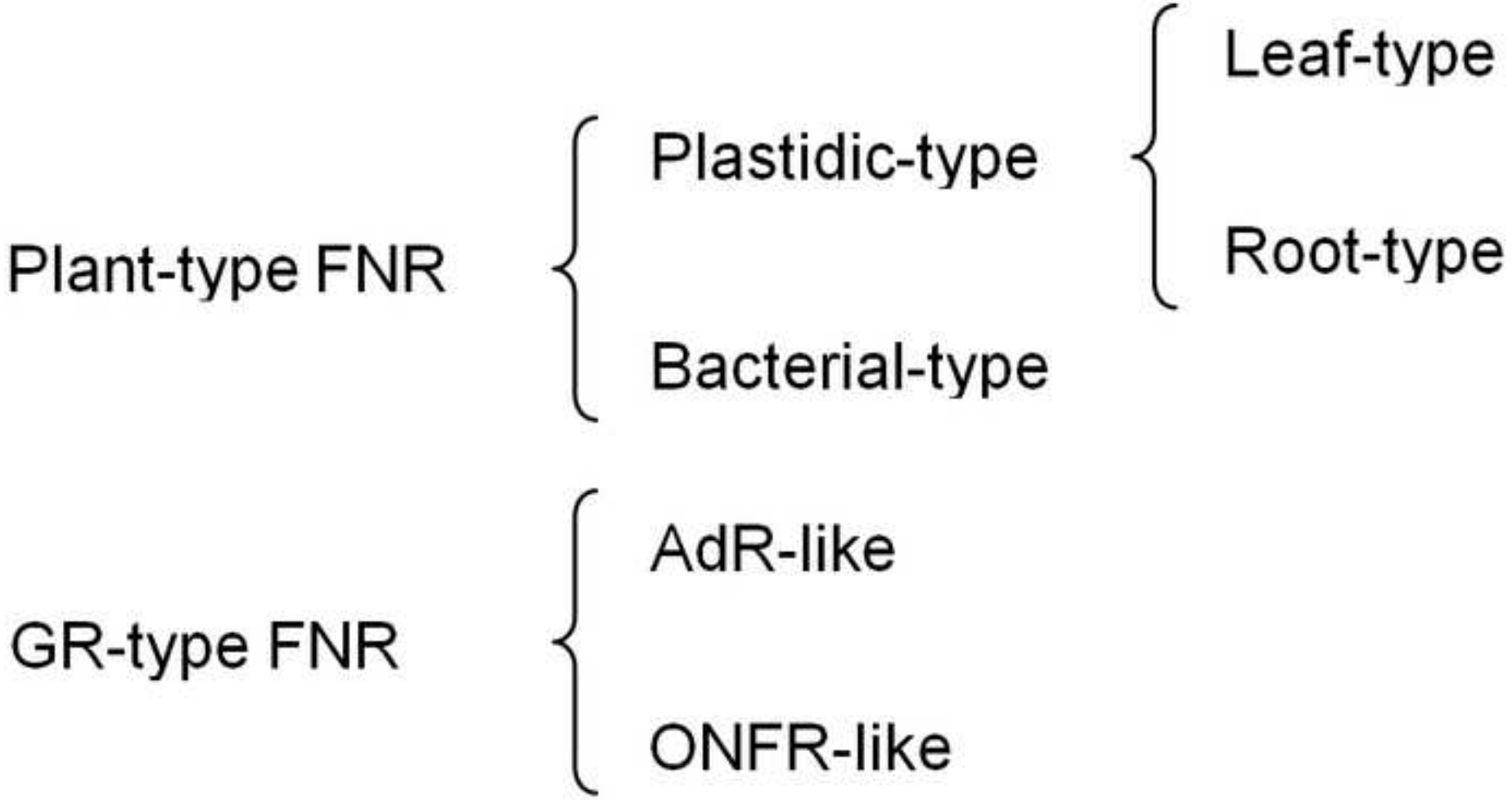

Fig_2

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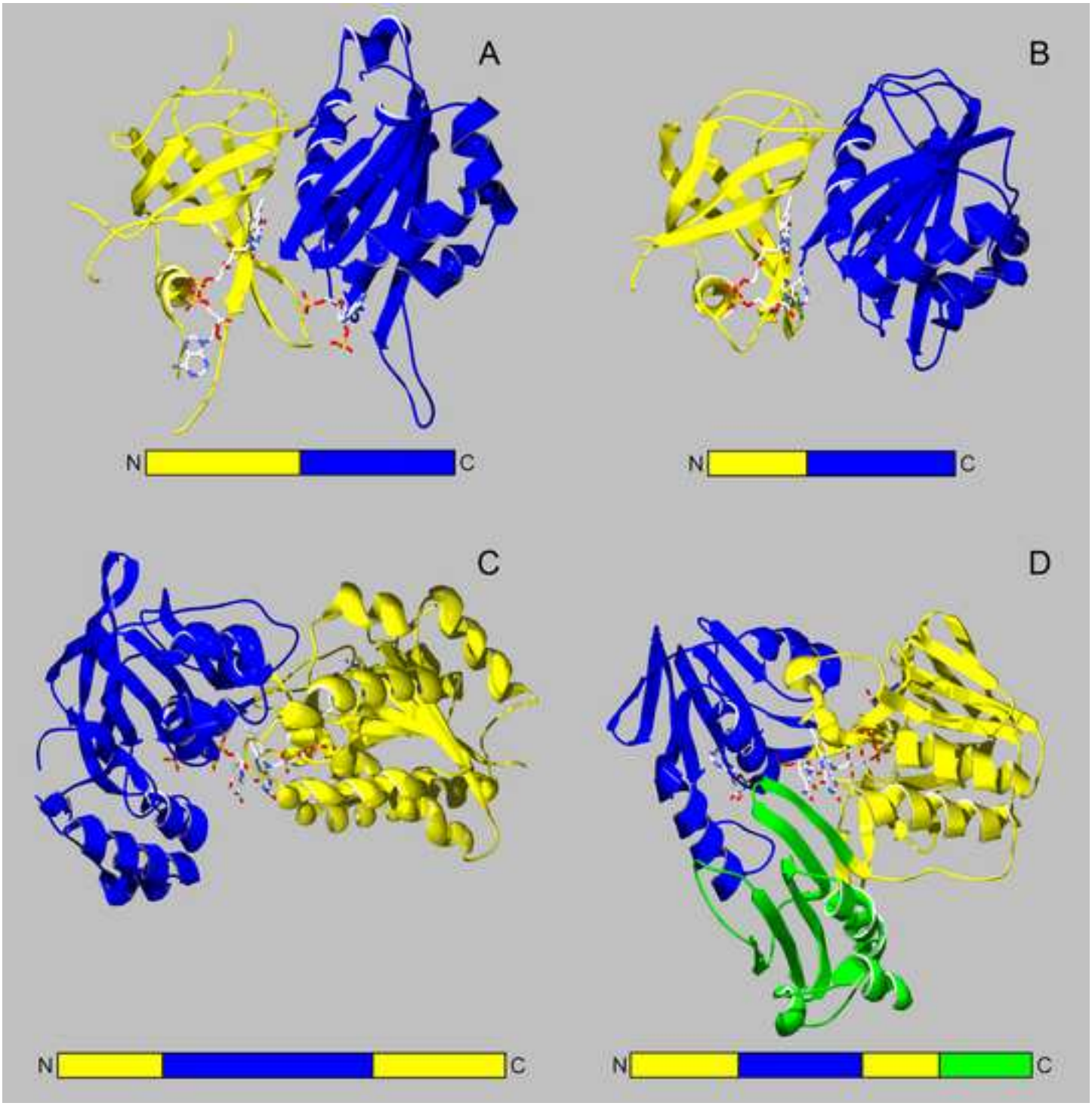

Fig_3

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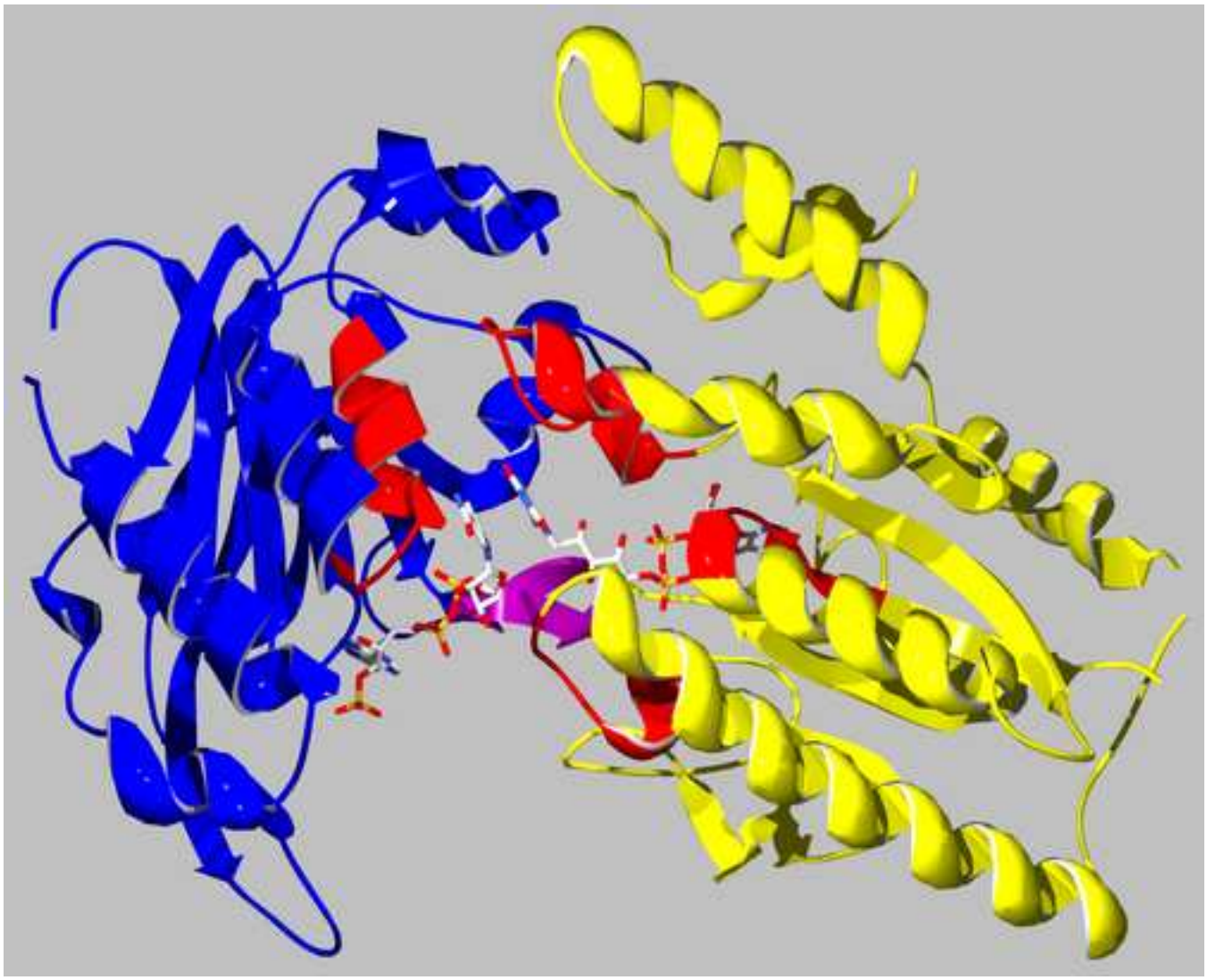

Fig_4

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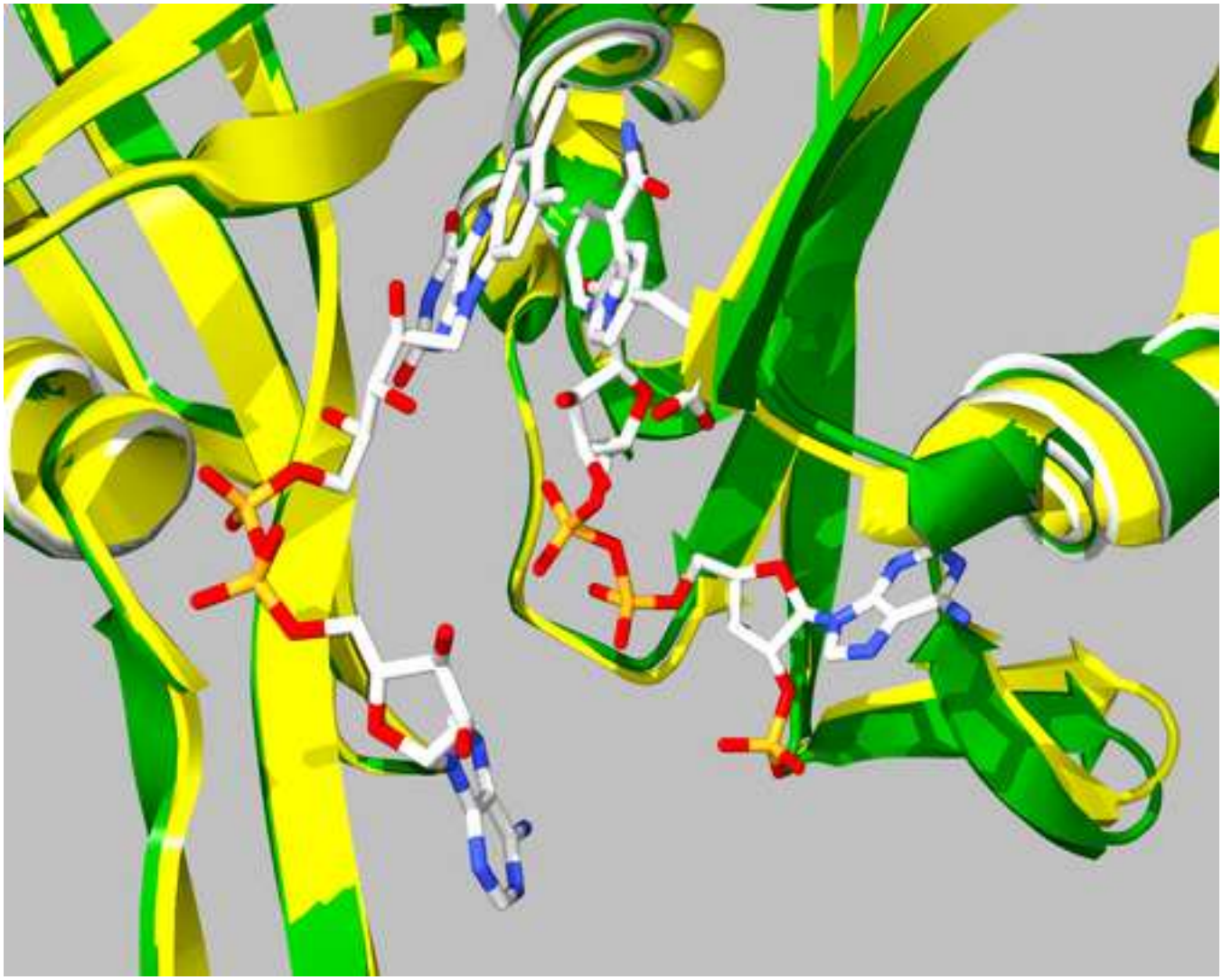

Fig_5

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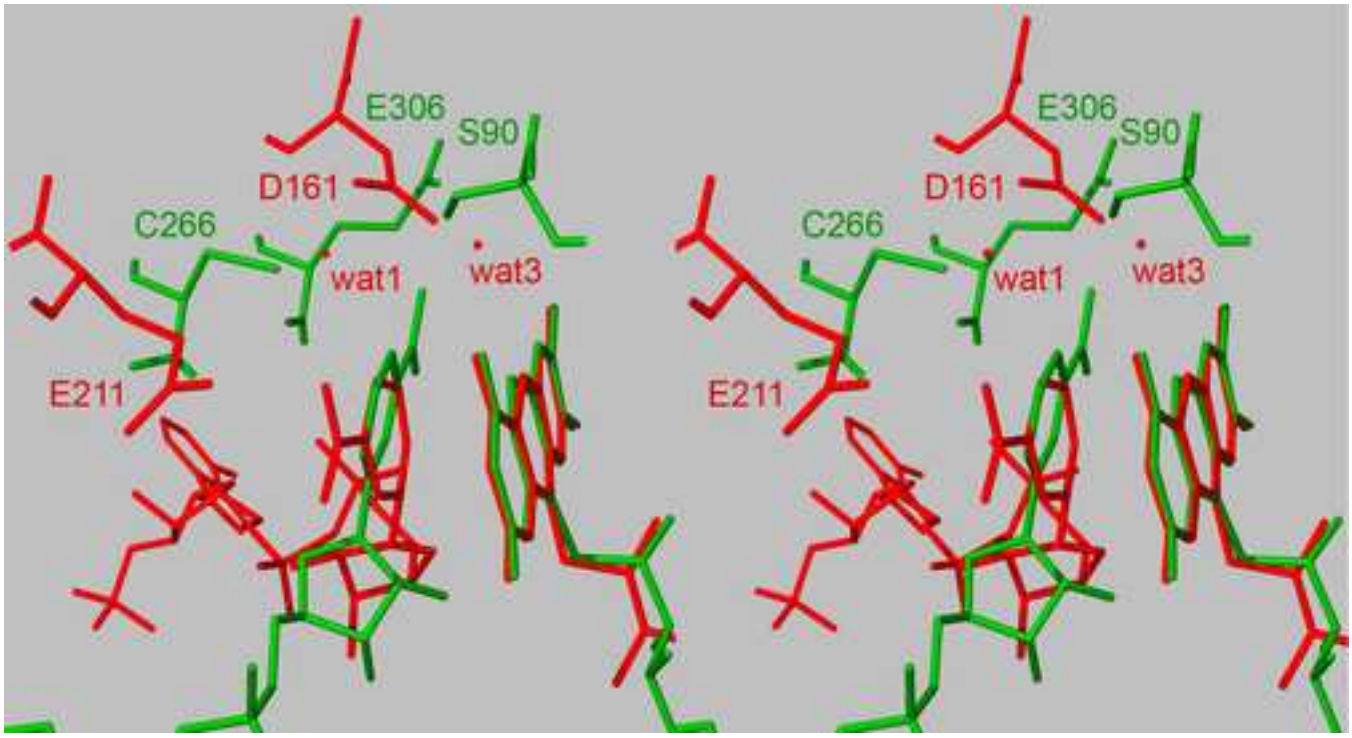

Fig_6
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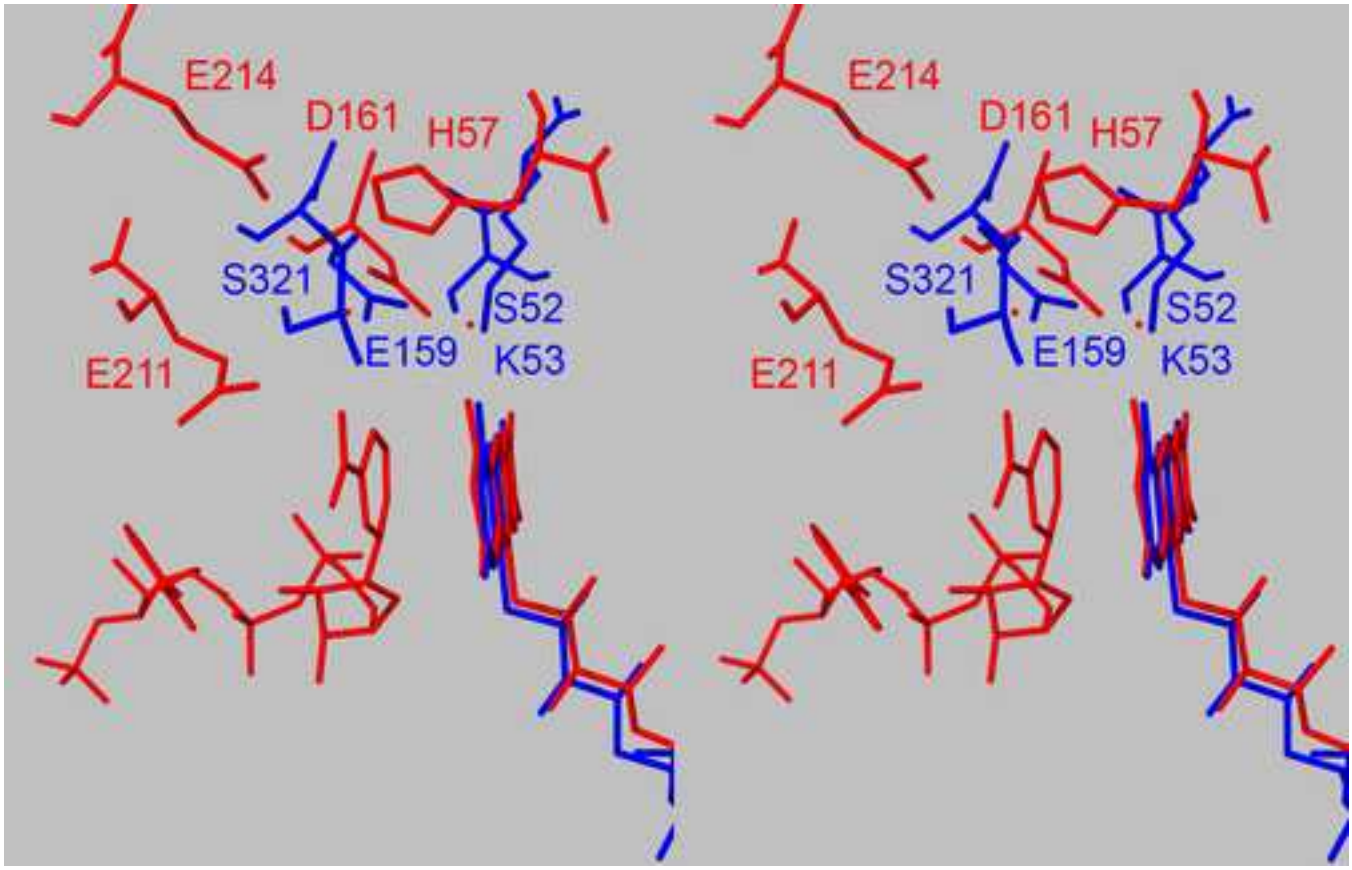

Fig_7

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