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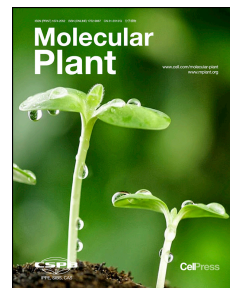
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A New Member Of The Thioredoxin Reductase Family From Early Oxygenic Photosynthetic Organisms*

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* Dedicated to the memory of David B. Knaff who made major contributions to the biochemistry of flavin enzymes and proteins engaged in redox regulation by thiol transitions.

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Dear Editor,

Thioredoxins (Trxs) are key components of the redox system that regulates the activity of a spectrum of target proteins through dithiol-disulfide exchange reactions. Trxs are reduced by members of the Trx reductase (TR) family (Jacquot et al., 2009). NADP-dependent Thioredoxin Reductases (NTRs), the most common type, belong to the family of dimeric pyridine nucleotide disulfide oxidoreductase flavoproteins that use NADPH as the source of reducing equivalents. In oxyphotosynthetic organisms, in particular, NTRs coexist with the ferredoxin/thioredoxin system (FTS), composed of ferredoxin (Fdx), ferredoxin:thioredoxin reductase (FTR) and a Trx. FTRs convert the electron signal obtained from photoreduced Fdx to a thiol signal via a 4Fe-4S center and a redox-active disulfide catalytic center. FTR, in turn, reduces Trx.

In cyanobacteria and chloroplasts, the FTS is closely associated with the regulation of enzymes of the Calvin-Benson cycle and associated processes—e.g., the oxidative pentose phosphate pathway (Balsera et al., 2014). In contrast to other oxygenic photosynthetic organisms, the ancient cyanobacterium *Gloeobacter* and the ocean-dwelling green oxyphotobacteria *Prochlorococcus* lack an FTR gene. This observation raises the question of how these photosynthetic organisms link the Calvin-Benson cycle and related metabolic processes to light and other changing environmental conditions.

To gain information on this point, we have conducted a comparative analysis of NTR-like Trx reductase protein sequences and identified an enzyme common to *Gloeobacter* and green oxyphotobacteria that possibly functions in this connection. The enzyme—provisionally named “DTR” for Deeply-rooted bacterial Thioredoxin Reductase—shows high similarity to NTR but exhibits unique structural features (Figure 1). The enzyme is present in other bacteria, including organisms within the deeply-rooted bacterial lineages as well as marine algae (Table S1; Figure S1). A multiple protein sequence alignment showed that *Gloeobacter* and *Prochlorococcus* DTRs harbor the conserved FAD-binding motif and the two active-site Cys residues typical of authentic NTRs (Figure 1A). Major differences were noted, however, in the amino acids responsible for pyridine nucleotide binding (GxGxxA/G and HRRxxR) (Figure 1A). These included substitutions of crucial residues for coordination to the pyrophosphate group and absence of specific positively charged amino acids needed for electrostatic interactions with the 2'-phosphate group of the

adenosine. Additional variations included a C-terminal extension with a conserved aromatic residue, and a fused N-terminal redoxin domain that is restricted to a few bacteria (grey box, Figure S1).

The visible-UV absorption spectrum of purified *Gloeobacter* DTR (GvDTR) showed features typical of a flavoprotein, including absorption maxima at 391 and 459 nm (continuous black line, Figure 1B) (Prongay and Williams, 1992). In contrast to NTRs, the *Gloeobacter* flavoprotein failed to show activity in the assay of the enzyme in which the oxidation of NAD(P)H is coupled to the reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Figure 1C) (Holmgren and Bjornstedt, 1995). The apparent lack of activity could be due to either of two reasons. One, NAD(P)H reduces FAD, but electrons are not further shuttled to its homolog Trx and thus to DTNB; nonetheless, the activity of *Gloeobacter* Trx in the coupled assay was demonstrated with *E. coli* NTR (Figure 1C). Alternatively, the flavoprotein does not function with NAD(P)H.

To explore the second possibility, we applied isothermal titration calorimetry (ITC) as the most direct approach for assessing protein-ligand binding. For these experiments, we included 3-acetylpyridine adenine dinucleotide phosphate (AADP), a non-hydrolyzable analog of NADPH, that binds tightly to NAD(P)-dependent enzymes. In contrast to *Escherichia coli* NTR (EcNTR) that binds NADP⁺ and AADP⁺ with a K_d in the micromolar range, the *Gloeobacter* protein failed to bind either ligand *in vitro* (Figure 1D and Figure S3). The lack of affinity for pyridine nucleotides resembles TR from the archaeon *Thermoplasma acidophilum* (TaTR; Hernandez et al., 2008), although structural differences were detected between the two proteins. GvDTR displayed a modified GxGxxA/G structural pattern for nucleotide binding (Figure S4, red box), and contained a C-terminal extension missing in TaTR (Figure S4, green box).

To confirm that the *Gloeobacter* flavoprotein fulfills the function of a TR, we determined whether it could reduce Trx using dithionite as artificial electron donor. Trx m (also known as TrxA) is the only canonical Trx form in *Gloeobacter* and *Prochlorococcus* (Balsera et al., 2014; Florencio et al., 2006). We observed that dithionite was able to slowly reduce the flavoenzyme anaerobically as measured by a decrease in absorbance at 459 nm (Figure 1B). Partial reoxidation of the enzyme was observed immediately after adding *Gloeobacter* Trx as seen by recovery of the main features of the visible-UV spectrum. These results

provide evidence that the *Gloeobacter* flavoprotein has Trx reductase activity and functions independently of pyridine nucleotides.

To gain information on the molecular properties of DTR, we determined a high-resolution structure of the *Gloeobacter* protein by X-ray crystallography at 1.9 Å resolution (PDB code 5J60; Table S2). The crystal structure revealed that GvDTR is a homodimer and that each monomer adopts a typical NTR fold (Figure 1E). Despite the high similarity between the FAD- and *pseudo* NADPH-binding domains with those of authentic NTRs, the relative orientation of the domains in the crystal structure differed from the flavin-oxidizing (FO) and flavin-reducing (FR) conformations found in NTRs (Lennon and Williams, 1997) (Figure S5). No direct interaction was observed between amino acids of the *pseudo* NADPH-binding domains, as detected between the two corresponding domains in the NTR dimer both in FO and FR conformations, suggesting that the two functional domains in DTRs are flexible relative to each other. FAD in GvDTR was found to be oxidized based on the planar conformation and the yellow color of the crystal. The redox-active Cys residues were reduced and distant from the *re* face of the isoalloxazine ring instrumental for electron transfer (Figure 1E).

Coordination to the FAD cofactor is well conserved compared to NTRs of known 3D structure (Figure S6). However, analysis of the NADPH-binding pocket revealed significant structural deviations from NTRs (Figure S7). One of the most striking variations is related to the substitution of the second strictly conserved Gly in the GxGxxA/G motif in EcNTR by an Asn in GvDTR (Figure 1A, red box). GvDTR structure showed that the substrate binding pocket is blocked by the side chain carboxamide of Asn (N154; Figure S7E), supporting the finding that the enzyme does not bind pyridine nucleotides.

Remarkably, the aromatic side chain of a tryptophan (W315) in one monomer stacks over the central portion of the FAD isoalloxazine ring of the second monomer on its *re* face (Figure 1E). This position is generally occupied by either the pyridine nucleotide or the disulfide bridge in NTRs. The tryptophan position is highly conserved in the protein sequences of the DTR family, including early branching bacteria, cyanobacteria and marine algae, replaced by a tyrosine residue only in firmicutes and chlorobi (Figure S1), and suggests a potential physiological role for this residue. As displayed here, the C-terminal tail has not previously been reported and is a unique feature of DTR. Relative to WT, deletion of the C-terminal motif in *Gloeobacter* DTR (GvDTR_Δt) was accompanied by

ca. a 7-9 nm shift in the flavin absorption maxima in the blue region of the spectrum (Figure 1B, lower panel). More interestingly, removal of the C-terminal tail increased the rate of flavin reduction by dithionite (~ 10-fold) to levels comparable to those with the *E. coli* enzyme (Figure 1B). Collectively, these results suggest that the unique C-terminal module on DTR has a direct influence on enzyme activity and might constitute a mechanism of regulation not previously reported.

The C-terminal extension of DTR resembles the C-terminal subdomain of certain ferredoxin-NADP reductase enzymes (FNRs) in which an aromatic residue protrudes over the *re* side of FAD, thereby requiring its displacement for a productive interaction between the pyridine nucleotide and FAD (Ceccarelli et al., 2004). Among the different types of FNRs, structural similarities were detected between DTR and TR-type FNRs. These FNRs harbor conserved FAD and NADPH-binding motifs but lack the redox active Cys. An aromatic residue in a short alpha-helix at the C-terminus protrudes over the *re* side of FAD (Figure S8). Mutational studies in TR-type FNR enzymes have shown that replacement of the aromatic residue on the *re* face of the flavin perturbs binding with its redox partner (Seo et al., 2014). Following a molecular mechanism similar to these FNRs, displacement of the C-terminal extension of DTR would allow access of the redox disulfide to the *re*-face of FAD likely in a regulatory manner. As for NTRs, conformational flexibility is expected to play an important role in the mechanism of enzyme action. Thus DTR in solution may exist in equilibrium between the structure observed in the crystal and other configurations in which the donor molecule (and/or the disulfide) is in close contact with the flavin group. In light of the high structural similarity with NTRs and the finding that DTR lacks the archetypal NADPH binding site, the physiological donor is thought to be a cofactor acting similar to pyridine nucleotides. Other types of molecules such as small redox carrier proteins can, however, not be excluded. To this point, a ferredoxin-linked flavoprotein enzyme that reduces Trx has been isolated from clostridia (Hammel et al., 1983). In the end, only the identification of the physiological electron donor will clarify the catalytic mechanism of enzyme reduction and its physiological importance in the Trx system.

In the present study, we have provided evidence for the diversity of Trx systems in bacteria and for variation in the ability of the reductases to deliver electrons to Trx. Our results support the view that the redox systems consist of structural modules and regulatory

elements that different organisms have conveniently combined during evolution to create proteins capable of adapting to specific metabolic and environmental situations. They also provide a structural foundation for future exploration of the mechanism of action and open the door to understanding the evolutionary development of different flavin reductases in relation to particular metabolic demands.

ACCEPTED MANUSCRIPT

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Figure 1. (A) Multiple sequence alignment of *Escherichia coli* NTR (EcNTR), *Gloeobacter violaceus* DTR (GvDTR) and *Prochlorococcus marinus* MIT9313 DTR (PmDTR) protein sequences. The motif for FAD binding (GxGxxG) and the redox active Cys (CxxC) are shown with black boxes. Red boxes indicate motifs for NAD(P)H binding (GxGxxA/G and HRRxxxR). At the C-terminal extension (green box), the tryptophan residue is marked with a triangle. Residue conservation colors are shown according to default ClustalX parameters; (B) Visible absorption spectra of EcNTR, GvDTR and GvDTR_Δt corresponding to band II, which is associated with the molecular environment and chemical state of the isoalloxazine moiety of the flavin. Flavoenzymes are represented by a black line. Red lines show the flavin absorption spectra after incubation with dithionite (DTH) under anaerobic conditions for the following times: 6 min (EcNTR), 70 min (GvDTR) and 8 min (GvDTR_Δt). The spectra obtained after addition of homologous Trx m to the reduced system (less than 3 min) are displayed as dashed black lines; (C) Absorbance changes at 412 nm as a consequence of DTNB reduction using NADPH as source of reducing equivalents for DTR. EcNTR that is active with its homologous Trx (EcTrx) as well as GvTrx m served as control; (D) A summary of dissociation constants (K_d), binding enthalpy (ΔH), and stoichiometry (n) of the flavoenzymes for pyridine nucleotides obtained from ITC experiments (Figure S3). K_d values obtained for *E. coli* NTR were comparable to those reported previously (Mulrooney and Williams, 1997). Relative error in K_d is 15 %; absolute errors in ΔH and n are 0.3 kcal/mol and 0.1, respectively; (E) Crystal structure of GvDTR homodimers (each monomer represented in yellow and green colors). Missing loops are represented as dashed lines (monomer 2 in green showed poorer electron density in some loop regions than monomer 1). The redox active Cys are reduced in the crystal and are shown in space-fill representation. The aromatic residue (W315) of the C-terminal tail (C-t tail) in one GvDTR monomer stacks over the FAD of the other monomer forming a pi-stacking (depicted as stick model). The inset shows a zoom-view of the tryptophan-FAD interaction.

Figure 1

