Ferrodoxin-NADP⁺ Reductase and Ferrodoxin of the Protozoan Parasite Toxoplasma gondii Interact Productively in Vitro and in Vivo*

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Toxoplasma gondii possesses an apicoplast-localized, plant-type ferrodoxin-NADP⁺ reductase. We have cloned a [2Fe-2S] ferrodoxin from the same parasite to investigate the interplay of the two redox proteins. A detailed characterization of the two purified recombinant proteins, particularly as to their interaction, has been performed. The two-protein complex was able to catalyze electron transfer from NADPH to cytochrome c with high catalytic efficiency. The redox potential of the flavin cofactor (FAD/FADH⁻) of the reductase was shown to be more positive than that of the NADP⁺/NADPH couple, thus favoring electron transfer from NADPH to yield reduced ferrodoxin. The complex formation between the reductase and ferrodoxins from various sources was studied both in vitro by several approaches (enzymatic activity, cross-linking, protein fluorescence quenching, affinity chromatography) and in vivo by the yeast two-hybrid system. Our data show that the two proteins yield an active complex with high affinity, strongly suggesting that the two proteins of T. gondii form a physiological redox couple that transfers electrons from NADPH to ferrodoxin, which in turn is used by some reductive biosynthetic pathway(s) of the apicoplast. These data provide the basis for the exploration of this redox couple as a drug target in apicomplexan parasites.

Toxoplasma gondii, the causative agent of toxoplasmosis, is a protozoan parasite belonging to the phylum Apicomplexa (1). This phylum comprises several other pathogens of humans or economically important animals, e.g. Plasmodium sp. (causative agent of malaria), Cryptosporidium parvum (cryptosporidiosis), and Eimeria sp. Nearly all Apicomplexa possess an unique organelle called apicoplast (for reviews see Refs. 2–4). Recent studies have established that it is related evolutionarily to plastids of photosynthetic red algae and that it was acquired by the ancestor of extant Apicomplexa by an endosymbiotic event (5, 6). The circular apicoplast genomes (35 kb in size) of both T. gondii and Plasmodium falciparum have been sequenced completely (7, 8). Their reduced size with respect to those of plant and algal plastids suggests that loss of genes encoding apicoplast proteins or their progressive transfer to the nucleus has taken place. Proteins to be imported into the organelle possess a characteristic N-terminal bipartite targeting sequence, which is both necessary and sufficient to transport these proteins into the apicoplast (4, 9–11). This distinct sequence feature has recently allowed the identification of some enzymes predicted to be localized in the apicoplast (10, 12–14) and helped in the assembly of a provisory metabolic map of this organelle by whole genome analysis (15).

Recently, both a ferrodoxin-NADP⁺ reductase (FNR)¹ of T. gondii and a [2Fe-2S] ferrodoxin(Fd) of P. falciparum have been cloned (16). They both contain a putative N-terminal apicoplast targeting signal that was shown in the case of T. gondii FNR (TgFNR) to be sufficient to target a reporter protein into the apicoplast (17). TgFNR starts from residue 150 of the whole coding sequence was expressed in Escherichia coli, and the enzyme was shown to be active as an NADP+-dependent oxidoreductase (16). Phylogenetic analysis indicated that TgFNR is more similar to the isoforms present in non-photosynthetic plastids than to those of cyanobacteria or chloroplasts. In the latter cases, FNR is responsible for the electron transfer from photosystem I to NADP⁺, according to Equation 1.

2Fd⁺ + NADP⁺ + H⁺ —> 2FdH⁺ + NADPH

(Eq. 1)

NADPH is used subsequently in the Calvin cycle (18). In non-photosynthetic plastids electrons flow in the reverse direction, from NADPH to Fd⁺, yielding FdH⁺ (19), which then serves as a reductant for various enzymes, i.e. nitrite reductase, glutamate synthase, sulfite reductase, and lipid desaturases (20, 21). By analogy, the redox system NADPH/FNR/Fd of the apicoplast would be predicted to provide reducing power to biosynthetic pathways yet to be identified. It is assumed that the apicoplast has retained a number of parasite-specific metabolic tasks, and because the vertebrate hosts of Apicomplexa do not possess a homolog of this organelle, these metabolic pathways are therefore prime targets for efficient and specific anti-infection drugs (22). In this respect the FNR/Fd redox system is a

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‡ The abbreviations used are: FNR, ferrodoxin-NADP⁺ oxidoreductase (EC 1.18.1.2); TgFNR, T. gondii FNR; 6H-L/TgFNR, His-tagged longer form of TgFNR; S/TgFNR, shorter form of TgFNR; SoFNR, S. oleracea leaf FNR; ZmFNR, Z. mays root FNR; Fd, ferrodoxin; TgFd, T. gondii Fd; PfFd, P. falciparum Fd; PfFNR, P. falciparum FNR; Fd⁺, oxidized Fd; FdH⁺, reduced Fd; NLS, nuclear localization signal; Ni-NTA, nickel-nitrilotriacetic acid; FPLC, fast protein liquid chromatography.
promising candidate, because it is presumably present in the apicoplast of most, if not all, apicomplexan parasites (23).

A detailed knowledge of the biochemical properties of apicomplexan FNR and its specific interaction with Fd is a prerequisite for the development of inhibitory substances of this redox couple. Here we report on the overproduction and purification of TgFNR, as well as T. gondii Fd (TgFd). Their subsequent biochemical characterization allowed us to highlight several distinctive differences of the parasite FNR/Fd redox couple compared with their plant counterparts, especially with regard to the FAD cofactor redox potential, the catalytic efficiency, and the protein-protein interactions.

EXPERIMENTAL PROCEDURES

Materials—All chemicals and pyridine nucleotides were purchased from Sigma-Aldrich. All chromatographic columns and media were purchased from Amersham Biosciences, with the exception of Ni-NTA-agarose, which was from Qiagen. The spinach (Spinacia oleracea) ferredoxin I (SoFdI), spinach FNR (SoFNR), and maize (Zea mays) root FNR (ZmFNR) were purified as reported previously (24–26).

DNA Constructs—For expression of TgFNR and TgFd, their mature coding sequence was placed under the control of the T5-based, isopropyl-1-thio-b-D-galactopyranoside-inducible hybrid promoter P<sub>lac</sub>lacUV5 from vector pDS65/RBSII (27), yielding plasmids pS1-TgFd and pS1-TgFNR, respectively (see Fig. 1). A commercial version of the above vector, pQE31 (Qiagen) served as recipient for a longer version of the coding sequence of TgFNR starting at Leu-121 (yielding pQE-L/TgFNR; see Fig. 1). The coding sequence of TgFd was taken from expressed sequence tag clone CG650907 (which is derived from mRNA of the VSG strain), and its insert was sequenced further and shown to encode all of the mature TgFd and to possess a long N-terminal extension but without an initiator AUG. A lexA-based yeast two-hybrid system was used for the detection of protein-protein interactions in vivo (28). The shorter TgFNR was cloned as an EcoRI fragment into pB42AD (Origene), and the different ferredoxins were cloned into the EcoRI site of pGILDA (Clontech). Plasmids pTUK-TpFd (16), pETFd (24), and pS1-TgFd (see above) served as source for the ferredoxins. In both two-hybrid plasmids expression is driven by a galactose-dependent promoter (see Fig. 1). All constructs were verified by DNA sequencing in the newly assembled parts of the vector. Sequence alignments were performed using Clustalall, and protein-protein interactions were visual inspected (29).

Overproduction of TgFNR and TgFd—E. coli RILM15 cells transformed with expression plasmids pTUK-TgFNR, pS1-TgFd, pQE-L/TgFNR, pQE-L/TgFd, pS1-TgFd were grown in flasks under vigorous shaking in 20 ml of Luria-Bertani (LB) medium supplemented with 100 μg/ml ampicillin. For protein purification, E. coli cells were grown at 37°C for 3 h (for TgFNR) or 2 h (for TgFd) of further growth.

Purification of TgFNR and TgFd—All purification steps were performed at 4°C except for FPLC, which was carried out at room temperature. E. coli cell paste was resuspended in 2.5 ml/g of Buffer A (50 mM sodium phosphate, pH 8.0, containing 300 mM NaCl, 10 mM imidazole, and 1 mM 2-mercaptoethanol supplemented with 1 mM phenylmethanesulfonyl fluoride and disrupted by sonication. After removal of cell debris by centrifugation at 43,000 × g for 1 h, the soluble fraction was loaded on an Ni-NTA-agarose column pre-equilibrated with Buffer A. The molarity of imidazole in Buffer A was brought to 20 mM and then used for extensive washing. Elution of recombinant proteins was then performed with 250 mM imidazole in Buffer A. In the case of TgFNR, the pooled enzyme-containing fractions were supplemented with 5 mM EDTA and dialyzed against 25 mM sodium phosphate, pH 7.0, containing 10% glycerol, 1 mM EDTA, and 1 mM 2-mercaptoethanol. TgFNR was then loaded on a HiLoad SP-Sepharose high performance column and eluted with column volumes of a linear gradient from 0.5 mM NaCl to 10% glycerol, 1 mM EDTA, and 1 mM dithiothreitol. In the case of TgFd, the protein eluted from the Ni-NTA-agarose column was concentrated by ultrafiltration and gel-filtered on a Superdex 75 column equilibrated in 150 mM Tris-HCl, pH 8.0, containing 1 mM NaCl and 10% glycerol. The purified TgFd was stored at –80°C under nitrogen in 150 mM Tris-HCl, pH 7.0, containing 10% glycerol.

Protein Characterization Methods—SDS-PAGE was performed on an Agilent 4543 diode-array spectrophotometer. The extinction coefficient of the protein-bound flavin was determined spectrophotometrically by quantitating the FAD released from the apoprotein following SDS treatment (30). Fluorescence measurements were performed on a Jasco FP-777 spectrophotofluorometer at 15°C. The identity of the enzyme-bound flavin was assessed fluorometrically with treatment with phosphodiesterase to release the flavin after thermal denaturation at 100°C (

Activity Assays—Enzyme-catalyzed reactions were monitored continuously on an Agilent 8453 diode-array spectrophotometer. Standard diaphorase activity was measured in 0.1 mM Tris-HCl, pH 8.2, at 25°C with K<sub>3</sub>Fe(CN)<sub>6</sub> as electron acceptor and NADPH or NADH as reductants. Standard cytochrome c reductase activity was assayed in the same buffer as above with either 7 or 9 μM SoFdI or TgFd, using 40 μM purified cytochrome c as the terminal electron acceptor. The NADPH concentration was kept constant by regeneration with 2.5 mM glucose-6-phosphate and 2 μg/ml glucose-6-phosphate dehydrogenase. Steady-state kinetic parameters for the Fd-dependent cytochrome c reductase activity were determined by varying the concentrations of both NADPH and Fd. Initial rate data (v) were fitted by non-linear regression using Grafit 4.0 software (Eyrthaus Software Ltd., Staines, United Kingdom) to a Michaelis-Menten equation.

Enzyme Photoreduction—All reduction experiments were carried out in an anaerobic cuvette at 15°C. Solutions were made anaerobic by successive cycles of equilibration with O<sub>2</sub>-free nitrogen and evacuation. Photoreduction of TgFNR using EDTA/light (31) were performed in 50 mM sodium phosphate, pH 7.0, containing 15 mM EDTA and 1.5 mM 5-carboxy-5-deazariboflavin.

Protein-Protein Interactions—To monitor the in vivo interactions between TgFNR and various Fds the protocol of Fashena et al. (28) was followed. Yeast strain EGY48 (MATa tyr1 his3 arg5 trp1 leu2-3;6;2 LeuA; Origene) containing the lacZ reporter plasmid pSH18-34 was used as recipient for all plasmids. As negative, non-interactive proteins two control plasmids from the supplier (Origene) were used. Interaction-dependent growth was assessed by plating the transformed cells onto His/Trp/Leu drop-out medium containing either 2% glucose or 2% galactose. Liquid β-galactosidase assays were performed in 96-well plates using Y-PER yeast lysate solution (Pierce) and o-nitrophenyl-β-D-galactopyranoside (Sigma) as enzyme substrate, following the instructions of the manufacturer (Pierce).

Protein cross-linking was performed as described previously (32). FNR and Fd (8 and 40 μM, respectively) were cross-linked by treatment with 5 mM N-ethyl-3(3-dimethylaminopropyl)carbodiimide in 25 mM sodium phosphate, pH 7.0. Affinity chromatography of FNrs was performed on Fd-Sepharose 4B. Immobilized SoFdI was obtained by coupling 250 nmoI of SoFdI to 350 μg of CNBr-activated-Sepharose 4B following the manufacturer's directions. SoFdI-conjugated resin (1 ml) was packed and used as an affinity column in an FPLC apparatus. FNrs (~10 nmoI) were loaded on the affinity column, equilibrated in 20 mM HEPES, pH 7.5, containing 10% glycerol and 1 mM dithiothreitol. After washing with the same buffer, FNrs were eluted with a linear gradient of NaCl. A second type of experiment was performed under the same conditions except for the presence of 2 μM urea in all the buffers and samples.

RESULTS

Production and Purification of T. gondii FNR—Several constructs were designed and utilized for the production of TgFNR in E. coli. The originally described plasmid pTUK-TgFd, which allows expression of TgFNR as a fusion protein with His<sub>6</sub>-tagged yeast ubiquitin, starting at Ser-150 of the cDNA-derived FNR sequence (16), was found unsuitable for high level purification. A second plasmid was constructed in which the above coding sequence contained only an N-terminal His<sub>6</sub> tag for purification (Fig. 1). Although for both plasmids the recombinant TgFNR was found soluble and active, the purified enzymes thus obtained were nevertheless prone to precipitation and underwent proteolysis during storage. Fur-
thermore, a small percentage (~4%) of the enzyme contained a modified flavin cofactor, which was identified as 6-OH-FAD (33). With the aim of stabilizing the protein, Leu-121 of TgFNR, which encompasses a region well conserved in non-photosynthetic FNRs (16), was chosen as the starting point for the longer mature form (Fig. 1). Again an N-terminal His6 tag was added to facilitate purification of the protein, resulting in plasmid pQE-L/TgFNR. Notwithstanding the low expression level obtained for this protein, a two-step procedure (affinity chromatography on Ni-NTA-agarose followed by ionic-exchange chromatography on SP-Sepharose) led to the production of 2 mg of purified enzyme/liter of culture (named 6H-L/TgFNR).

**Molecular Properties of 6H-L/TgFNR**—6H-L/TgFNR migrates in SDS-PAGE as a band of ~43 kDa (see Fig. 6, lane 5). The molecular mass value of the native enzyme as analyzed by FPLC on a Superdex 75 column was 43 kDa, well in keeping with that calculated from the deduced amino acid sequence (43.4 kDa, including the His6 tag). The gel filtration conditions were chosen to avoid possible aggregation phenomena, i.e. 10% glycerol and 1 mM dithiothreitol were added to the buffer. In fact, there are eight cysteines present in the deduced sequence of TgFNR. By treatment of the enzyme with 5,5'-dithiobis(2-nitrobenzoate) under denaturing conditions, all the cysteines were titrated, indicating that no disulfide bonds are present, whereas modification of 6H-L/TgFNR under non-denaturing conditions yielded only three readily titratable cysteines. This suggests that these latter residues are exposed to solvent and hence they could possibly form intermolecular disulfide bonds by oxidation, resulting in protein aggregation.

**Spectral and Redox Properties of 6H-L/TgFNR**—The absorption spectrum of the purified protein is that typical of a flavoprotein, with bands centered at 395 and 454 nm (33). Maximal absorbance in the UV region was at 272 nm. A value of 6.4 for the 

$A_{272}/A_{454}$ ratio was calculated from the spectrum. Flavin fluorescence was quenched almost completely. The non-covalently-bound flavin in 6H-L/TgFNR was shown to be FAD. The extinction coefficient of the enzyme at 454 nm was calculated to be 10.1 mM$^{-1}$ cm$^{-1}$ from the amount of FAD released after protein denaturation by SDS.

We explored the anaerobic reduction of the flavin cofactor bound to the enzyme to obtain information about its redox potential and the intermediate species formed during reduction. Stepwise anaerobic photoreductions of 6H-L/TgFNR were performed for the free enzyme (Fig. 2A) and in the presence of an equimolar amount of NADP$^+$ (Fig. 2B). During reduction, the neutral blue semiquinone accumulated, as is the case for the plant FNRs. When NADP$^+$ was present in the anaerobic cuvette, long wavelength bands absorbing well beyond 700 nm, which are ascribed to charge transfer species between NADP(H) and FAD(H$^-$), were formed. Data obtained in this latter type of experiments were used to calculate the concentrations of NADPH and fully reduced flavin at several photoreduction times. These results are plotted in Fig. 3, and data obtained in the same type of experiments for the spinach leaf and the maize root enzymes are added for comparison. The course of reduction in the case of 6H-L/TgFNR highlighted that nearly full reduction of the FAD cofactor ensued before NADPH started to accumulate significantly. This indicates that the value of the enzyme redox potential is more positive than that of the NADP$^+$/NADPH couple. Furthermore, comparison with the data obtained in the case of the two plant FNRs shows that 6H-L/TgFNR has a quite distinctive behavior; its pattern was nearly exactly specular with respect to that of the photosynthetic FNR, whereas the non-photosynthetic plant FNR showed an intermediate behavior. Shifting of the curve from left to right in this type of plot indicates that the redox potential of the enzyme FAD cofactor becomes less negative going from the photosynthetic to the non-photosynthetic isoform and
to TgFNR. This is in keeping with the different physiological roles of the different isoforms.

**Cloning and Expression of T. gondii Fd**—The physiological activity of the plant homologs of TgFNR is to catalyze electron transfer between NADP(H) and a [2Fe-2S] ferredoxin. To test for such activity we initially used FdI of spinach chloroplasts (photosynthetic isoform, SoFdI) because of difficulties in the production of substantial amounts of recombinant *P. falciparum* Fd described earlier (16). More recently, however, we could identify a single expressed sequence tag clone in the *T. gondii* expressed sequence tag database that encoded the whole mature TgFd sequence, evidenced by its high sequence identity to other plant-type Fds (Fig. 4, A and B) and part of the N-terminal apicoplast targeting domain (data not shown). The latest release of ToxoDB 2.0 (ToxoDB.org) contains a genomic contig (TGG_640), which encompasses the whole *T. gondii* Fd sequence including its complete N terminus with a predicted signal peptide (data not shown).

The putative mature form of TgFd was expressed as an His₆ tag fusion protein (see Fig. 1). Analysis of *E. coli* cell extracts revealed that only part of the synthesized TgFd was recovered as the holoprotein in the soluble fraction. The purification procedure required a second step after the Ni-NTA affinity chromatography. Gel filtration on Superdex 75 yielded a homogeneous holoprotein as judged by native PAGE and with a molecular mass of ~20 kDa by SDS-PAGE (see

**Fig. 2. Photoreduction of 6H-L/Tg-FNR.** Absorbance spectra of anaerobic 6H-L/TgFNR solutions recorded at successive stages of reduction are shown. Spectra were not corrected for the spectral contribution of 5-carba-5-deazariboflavin. The insets show an enlargement of the absorbance changes in the 600- and 750-nm regions due to the flavin semiquinone and charge-transfer complexes with NADP(H), respectively (35). A, photoreduction time course of 6H-L/TgFNR. B, photoreduction of the enzyme performed in the presence of an equimolar amount of NADP⁺.

**Fig. 3. Photoreduction of different FNR forms in the presence of NADP⁺:** relationship between NADP⁺ and FAD reduction. Absorbance at 340 nm, which is practically an isosbestic point for the various reduction forms of FNR, is used to monitor NADPH formation. At each time of irradiation, the ΔA₃₄₀, as a fraction of the maximal ΔA₃₄₀, was plotted against the ratio of fully reduced enzyme FAD to the sum of fully reduced and oxidized enzyme flavin. The data were corrected for the presence of the FAD semiquinone. ●, SoFNR; □, ZmFNR; ○, 6H-L/TgFNR.
A. Protein sequence comparison of different Fds. A, amino acid sequence alignment, of the mature part of different plant-type Fds. As a starting point for the alignment residues 3 of Anabaena variabilis was used, so that for all other Fds the N-terminal targeting sequences were omitted. Identical residues are shaded. The four Cys residues implicated in [2Fe-2S] binding are drawn in white with black shading, whereas residues that are charged in PfFd but uncharged in TgFd and SoFdI are indicated by an asterisk. The boxed residues are charge reversals between the two apicomplexan Fds and SoFdI. The protein accession numbers are as follows: A. variabilis, SP CA32528; Chlamydomonas reinhardtii, SP F03983; S. oleracea leaf; SP P00221; Z. mays root; SP BAA19250; P. falciparum, GB CAD5335; T. gondii GB AD489783. B, sequence comparison between different Fds. The percentage of identical residues is reported.

Fig. 4. Protein sequence comparison of different Fds. A, amino acid sequence alignment, of the mature part of different plant-type Fds. As a starting point for the alignment residues 3 of Anabaena variabilis was used, so that for all other Fds the N-terminal targeting sequences were omitted. Identical residues are shaded. The four Cys residues implicated in [2Fe-2S] binding are drawn in white with black shading, whereas residues that are charged in PfFd but uncharged in TgFd and SoFdI are indicated by an asterisk. The boxed residues are charge reversals between the two apicomplexan Fds and SoFdI. The protein accession numbers are as follows: A. variabilis, SP CA32528; Chlamydomonas reinhardtii, SP F03983; S. oleracea leaf; SP P00221; Z. mays root; SP BAA19250; P. falciparum, GB CAD5335; T. gondii GB AD489783. B, sequence comparison between different Fds. The percentage of identical residues is reported.

The kinetic parameters for the reductase reaction catalysed by 6H-L/TgFNR are shown in Table I. TgFd showed the typical spectrum of a [2Fe-2S] ferredoxin with peaks at 277, 333, 424, and 464 nm (data not shown). A value of 0.60 for the 277 to 321 nm ratio was determined.

Catalytic Properties of 6H-L/TgFNR—As expected, 6H-L/TgFNR was very active as an NADPH-dependent diaphorase. The kinetic parameters for the ferricyanide reductase reaction catalysed by different FNR forms are shown in Table II. The coupling to TgFd was stronger than that for leaf SoFdI, and there was a strong preference for NADPH over NADH shown even at 2 mM NADH. An approximate value for NADPH preference over NADH was 180,000-fold. The kinetic parameters for the NADPH-cytochrome c reductase activity of 6H-L/TgFNR with both SoFdI and TgFd are compared in Table I. Clearly, there is a preference of 6H-L/TgFNR for the parasite iron-sulfur protein. We also determined the kinetic parameters of plant FNR isozymes using TgFd as the protein substrate to evaluate the specificity of the protein-protein interactions in these various reductases (Table I). Apparently, 6H-L/TgFNR showed a 3-fold value half of that of the plant enzymes when TgFd was used as electron acceptor and even lower when SoFdI was used. On the other hand, the Km value of 6H-L/TgFNR for TgFd was about 3-fold lower than that for leaf SoFdI (Table I). Thus, the catalytic efficiency of the T. gondii couple was twice that of the leaf homolog (32 e- eq/s μM; see Ref. 34) or of the heterologous couples (Table II). SoFdI showed the same efficiency with both ferredoxins in this type of reaction.

Binary Complexes of TgFNR with Ferredoxins—Because electron transfer to Fd relies absolutely on the physical interaction between Fd and FNR, we wished to investigate this aspect in more detail. To date all protein-protein interaction studies between Fds and FNRs in any system have only been performed in vitro. To extend the experimental possibilities to in vivo, we established a yeast two-hybrid system consisting of S/TgFNR (note that this is the shorter version; see Fig. 1) and either SoFdI or the two apicomplexan Fds (Fig. 1). In the used two-hybrid format both genes are under control of a galactose-inducible promoter so that a potential interaction can only be observed under inducing conditions. Using this system and leucine auxothrophy as marker for a positive protein-protein interaction we observed that S/TgFNR can interact with all three Fds in vivo, although seemingly to a different extent as judged by the difference in cell mass on selective medium (Fig. 5A). According to this experiment, SoFdI bound better to S/TgFNR than did TgFd, and PfFd reproducibly in-
interacted only weekly. Using a second reporter as a measurement for interaction strength we determined the $\beta$-galactosidase activity of individual yeast clones after cross-mating them to a strain containing a lexA-driven lacZ reporter plasmid. Again SoFdI appeared to be more efficient in interacting with S/TgFNR than did TgFd or PfFd (Fig. 5B). This was not because of an increased amount of SoFdI produced by the yeast cells (or likewise a decreased production of PfFd) as judged by Western blot analysis using an antibody directed against the shared lexA part of the different fusion proteins (data not shown), implying that the differences reflect altered binding efficiency.

We further investigated these protein-protein interactions in the different redox systems (Apicomplexa and plants) using several in vitro approaches. The aim was to enhance our understanding of the T. gondii redox couple by exploiting the well-known properties of the plant counterparts. The spinach binary complex has substantially lower protein fluorescence than the isolated proteins (35). Titration of 6H-L/TgFNR with either TgFd or SoFdI resulted in quenching of the reductase fluorescence, reaching a final point at a 1:1 protein ratio. Fluorescence quenching by SoFdI (43%) was higher than that obtained by TgFd titration (30%), whereas similar values of $K_d$ were determined ($<20$ nM at 10 mM ionic strength). In the case of spinach FNR, the same fluorescence quenching was produced by either ferredoxins, but the binding affinity of TgFd to the heterolo-
Fig. 6. Carbodiimide promoted cross-linking of spinach leaf and *T. gondii* FNRs with Fds as analyzed by SDS-PAGE. The positions of the different purified proteins are indicated on the right of the gel. Lane 1, molecular mass markers (mass values in kDa are listed); lane 2, SoFNR + SoFdI; lane 3, SoFNR + SoFdI (after 30 min of incubation); lane 4, 6H-L/TgFNR + SoFdI (after 30 min of incubation); lane 5, 6H-L/TgFNR + TgFd; lane 6, 6H-L/TgFNR + TgFd (after 30 min of incubation); lane 7, SoFNR + TgFd (after 30 min of incubation). Protein bands corresponding to the cross-linked proteins are indicated by an asterisk.

Carbodiimide-promoted cross-linking has been exploited in the past to study protein-protein interactions, because it allows the isolation and characterization of the covalent complex thus obtained. We used a soluble carbodiimide to verify whether the *T. gondii* binary complex was mediated by ionic interactions between carboxyl and amino groups of the protein partners as reported for the spinach leaf redox couple (32). Whereas SoFdI yielded a binary covalent complex with both the spinach reductase and 6H-L/TgFNR, surprisingly TgFd was much less prone to undergo cross-linking with either reductases (Fig. 6).

We finally analyzed the interaction of SoFdI with the two different reductases using affinity chromatography on SoFdI-conjugated resin. In separate experiments both spinach FNR and TgFNR bound to the resin at low ionic strength in the presence or absence of 2 M urea. A chimeric protein comprising SoFdI and SoFNR obtained by gene fusion (36), run as a negative control, did not bind to the resin. Fig. 7 shows the chromatograms obtained when a linear gradient of NaCl was used to elute the enzyme from the affinity column. 6H-L/TgFNR eluted at a lower ionic strength than the spinach enzyme (Fig. 7A), indicating a stronger interaction of the latter. When 2 M urea was present in the equilibration buffer, both reductases were released from the affinity column at a lower NaCl concentration but this time in the reverse order; 6H-L/TgFNR now eluted at a higher ionic strength than did the leaf FNR (Fig. 7B). This behavior suggests that there are differences in the type of interactions that stabilize these protein complexes.

**DISCUSSION**

The main aim of these studies was the biochemical characterization of the FNR/Fd redox couple of *T. gondii* as a prerequisite for its exploitation as a drug target in apicomplexan parasites. This protein system is of plant origin and thus absent in the mammalian host. In mitochondria of eukaryotes including Apicomplexa, a redox system exists that is functionally similar to the FNR/Fd couple and consists of the adrenodoxin reductase-adrenodoxin system (23, 37). However, these reductases do not show any significant sequence or structural similarities to plant-type FNRs (37, 38). Here we have shown that the recombinant *T. gondii* proteins possess the characteristic features of the plant isoforms as far as absorption spectrum, cofactor content, and catalytic activities are concerned. In the discussion that follows, we will focus on those properties that differ.

One very important difference between the parasite enzyme and plant FNRs is related to the redox potential of the FAD prosthetic group, which has been tuned for the physiological direction of electron transport. During anaerobic photoreduction of the enzyme in the presence of an equimolar amount of NADP+ we found that the behavior of the *T. gondii* redox system was opposite to that of the photosynthetic FNR, i.e. in the case of SoFNR NADP+was reduced first, whereas in the case of TgFNR FAD was nearly fully reduced before NADPH accumulated. An approximate value of ~290 mV could be calculated for the redox potential \( E_{\text{redox}} \) of TgFNR, compared with ~340 mV for SoFNR (39). This is in keeping with the physiological direction of electron transport in these redox systems, from Fdred to NADP+ in photosynthetic tissues and from NADPH to Fdred in non-photosynthetic organelles.

Tuning of the FAD redox potential by the apoprotein has already been shown for the maize root ZmFNR (see Fig. 3 and Ref. 26). However, the adjustment for function in the case of TgFNR is by far more impressive. It can be envisaged that the redox potential of TgFd will be more positive than ~290 mV to facilitate electron transfer to the presumed reductive biosynthetic pathways of the apicoplast. At present we can only speculate what these might be. It has been proposed recently (23, 40) that the apicoplast might contain the core enzymes for [Fe-S] cluster biosynthesis and that Fd/FNR might fulfill the same essential task in this process as the functionally similar adrenodoxin/adrenodoxin reductase system in mitochondria, namely to provide electrons at various steps in [Fe-S] biogenesis (23, 41). In addition, a role in glutamate synthesis and/or lipid desaturation, both of which are known to be Fd-dependent in plant plastids (20, 21), cannot be ruled out at present, although direct evidence for the respective apicoplast-localized enzymes by analysis of the whole *Plasmodium* genome is still lacking (15). Finally, metabolic processes not found in plant plastids but unique for the apicoplast might exist.

Quite unexpectedly, TgFNR, which now undoubtedly has been shown by us to belong to the non-photosynthetic isomeric class of FNRs, did not possess a disulfide bond, a peculiar
feature of this class (26). Most likely, the disulfide has only a structural role in the plant enzymes, and it may be that the apicoplast environment is not suited to keep it in the oxidized form.

Overall, the interaction of TgFNR with NADP(H) is similar to that of plant FNRs, which is in keeping with the high similarity of the NADP binding domain. Only two short insertions are present in the TgFNR sequence just after the motif GTGVAP (16). In contrast to that the interaction of TgFNR with its protein substrate Fd shows distinctive features, which could be because of the substantial sequence differences of the FAD binding domains between plant and apicomplexan FNRs. A long insertion of 27 amino acids is present next to the residues implicated in Fd binding (25, 32) and just before the motif RYIS, which surrounds the active site isosaloloxazine ring of FAD (38). Several plasmidia FNRs found in the sequence databases possess similar insertions at the same position, albeit of different length and sequence. In a study of the interaction of TgFNR with NADP(H), it was found that the Kd values measured in vitro using the yeast interaction trap (45). The affinity between spinach Fd and FNR lies in the range of 10^{-7} to 10^{-8} M (21), which would require the most sensitive yeast strain to allow growth within 2–3 days (which is what we observe for the apicomplexan Fds). The stronger interaction observed in vivo between TgFNR and SoFdI compared with TgFd may be the result of the yeast nuclear environment, which probably is quite different from that of the apicoplast. The successful implementation of the FNR/Fd interaction trap was not anticipated per se for the following reason. Fd is supposedly not able to enter the yeast nucleus in sufficient quantity (because it lacks a nuclear localization signal; NLS) unless it interacts with FNR (which carries a vector-derived NLS; see Fig. 1). TgFNR, on the other hand, requires a fully assembled [2Fe-2S] cluster as a prerequisite for proper interaction with FNR. Because in yeast and other eukaryotes all [Fe-S] clusters seem to originate from mitochondria (41) this in turn would mean that a delicate balance in timing between synthesis of Fd in the yeast cytosol, subsequent assembly of the [2Fe-2S] cluster, interaction with FNR, and final transport of the complex into the nucleus (where transcriptional activation of the reporter takes place) would be required to detect the interaction between Fd and FNR with this system. Support for this assumption comes from the fact that the interaction trap in the reverse setup (i.e. Fd with vector-derived NLS and TgFNR without it) does not lead to any detectable transcriptional activation of the two reporters (data not shown), which could be explained by the fast delivery of NLS-Fd into the nucleus, and thus insufficient assembly of [Fe-S] clusters into it. An immediate application of the FNR/Fd interaction trap with regard to this redox pair being a potential drug target would be to screen for low molecular mass substances or peptides able to disrupt this protein-protein interaction specifically (for review of this approach see Ref. 46).

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