The Three-dimensional Structure of Two Redox States of Cyclophilin A from Schistosoma mansoni

EVIDENCE FOR REDOX REGULATION OF PEPTIDYL-PROLYL CIS-TRANS ISOMERASE ACTIVITY

Received for publication, March 29, 2007, and in revised form, June 21, 2007 Published, JBC Papers in Press, June 25, 2007 DOI 10.1074/jbc.M702714200

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Treatment of schistosomiasis, a widespread human parasitic disease caused by the helminth parasites of the genus Schistosoma, relies mainly on one chemotherapeutic agent, praziquantel, although several other compounds exert antiparasitic effects. One such compound is the immunosuppressant cyclosporin A, which has been shown to significantly diminish worm burden in mice infected with Schistosoma mansoni. Given the well established interaction between cyclosporin A and the cyclophilin superfamily of peptidylprolyl cis-trans isomerases, we solved the structure of cyclophilin A from S. mansoni (SmCypA) by x-ray crystallography in the reduced and oxidized states at 1.5 and 1.8 Å of resolution, respectively. Oxidized SmCypA contains a disulfide bridge between two C-terminal cysteines (Cys-122 and Cys-126). This is the first example of a cyclophilin containing this disulfide bridge. Parallel functional studies suggest a mechanism for regulation of SmCypA activity via oxidation of its thiol groups; in fact, whereas oxidized SmCypA is inactive, reduced SmCypA is an efficient isomerase active at nanomolar levels with a $k_{cat}/K_m$ of $1.1 \times 10^7 \text{M}^{-1} \text{s}^{-1}$, and it is inhibited by cyclosporin A (IC$_{50}$ of 14 ± 4 nM). The lack of conservation of this cysteine couple within the CypA superfamily, their close proximity to the active site, and the importance of thiol groups for peptidyl-prolyl cis-trans isomerase activity render this structural feature a challenge for the development of alternative and more effective anti-schistosomiasis inhibitors and may in addition imply an alternative function of SmCypA in the schistosome.

Cyclophilins (Cyps)$^2$ are ubiquitous cytosolic proteins that carry out a wide range of functions attributed to their PPIase activity, e.g. protein folding and chaperoning (1–3); they also participate in the regulation of intracellular signaling and have roles in T-cell activation and human immunodeficiency virus infection (4, 5). The immunosuppressant cyclosporin A (CsA) is a specific inhibitor of Cyp, and it has permitted an improved understanding of the roles adopted by Cyps; in particular, the Cyp-CsA complex exerts its well known immunosuppressive effect through an interaction with the protein phosphatase calcineurin (6–8).

Cyps are classified into isoforms according to their cellular localization and amino acid sequence conservation. The repertoire of Cyp functions is increasing with the identification of numerous atypical Cyps, including a 33-kDa human nuclear E isoform (hCYP33), which contains an additional RNA binding domain (9), and cyclophilins that contain regions involved in hormone receptor binding. In most species isoform A, which is found in abundance in the cytosol, is the major isoform; the less-abundant isoform B is located in the endoplasmic reticulum. However, in schistosomes, isoform A represents the minor species and was reported to exhibit decreased PPIase activity in comparison to isoform B (10).

At present, schistosomiasis, which infects 200 million people worldwide, is treated using the drug of choice, praziquantel (11). It is an obvious risk to base treatment on a single drug, and low sensitivity strains (11) have already appeared, making it clear that alternative treatments must be sought. Interestingly, in addition to its well known immunosuppressive effects, CsA (7) proved to have anti-parasitic activities against a number of human protozoal and helminth parasites, including schistosomes (12). Although the mechanisms of such effects are as yet unknown, they have been observed to be unrelated to the PPIase and T cell activation roles and, therefore, raise the possibility of cyclophilins possessing alternative functions in parasites (13).

To gain insight into the structure and role of SmCypA in the schistosome and to clarify its interaction with CsA, we solved

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1 This work was supported by Progetto Ateneo 2003 (University of Rome “Sapienza”), by the Consorzio Interuniversitario Biotecnologie (Urbino, Italy), and by the Ministero dell’Istruzione dell’Università e della Ricerca, FIRB 2003 “Biologia strutturale post-genomica: sviluppo di infrastrutture per la cristallografia delle proteine.” The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 2CMT and 2CK1) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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2 The abbreviations used are: Cyp, cyclophilin; CsA, cyclosporin A; hCYP33, 33-kDa human cyclophilin E; NEM, N-ethylmaleimide; PPIase, peptidylprolyl cis-trans isomerase; SmCypA, cyclophilin A from S. mansoni; Trx, thioredoxin; r.m.s.d., root mean square deviation; PEG 5K MME, polyethylene glycol 5000 monomethyl ether; TFE, trifluoroethanol.
the tertiary structure of SmCypA by x-ray crystallography. SmCypA contains seven cysteines; two of these (Cys-122 and Cys-126), located in close proximity to the active site, are not conserved in the Cyp superfamily as a doublet. We crystallized two oxidation states of the apoenzyme; one presenting the reduced cysteine couple, and the other containing a disulfide bridge between these two residues never reported before in other Cyps. We have carried out experimental experiments which demonstrate that these two diverse redox states have different PPIase activities; we proved that the oxidized form is inactive, whereas the reduced form is fully active. Given the presence of redox active cysteines and previous reports suggesting that the anti-parasitic actions of CsA are unrelated to the inhibition of PPIase activity (14), it is possible that in the schistosome SmCypA may have an alternative role that may stimulate the development of novel anti-schistosomiasis therapies, specific for this unique cysteine couple.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of SmCypA—The SmCypA gene (accession number U50388 (15)) was amplified from adult Schistosoma mansoni cDNA (a kind gift from Prof. R. Pierce, Institut Pasteur, Lille, France) using KOD HotStart DNA polymerase (Novagen) and gene-specific primers according to standard protocols. All reactions were carried out in a PTC-150 Minicycler™ (MJ Research Inc.). The gene was directionally cloned into the pGEX-4T-1 (GE Healthcare) expression vector via BamHI and XhoI restriction sites. The fidelity of the PCR reaction was confirmed by sequencing (BMR Sequencing Service, University of Padova, Padova, Italy).

SmCypA was expressed with an N-terminal glutathione S-transferase tag in BL21 (DE3) pLysS bacterial cells upon induction with 1 mm isopropyl-β-D-1-thiogalactopyranoside, incubating overnight at 22 °C. Successful expression was confirmed by SDS-PAGE, highlighting a band at ~45 kDa. After centrifugation, the bacterial cells were resuspended in 1× phosphate-buffered saline, pH 7.4, containing 0.25 mg/ml lysozyme and lysed by sonication (5 cycles of 45-s on, 15-s off pulses). The protein was purified from the soluble fraction by gravity flow on a disposable PD-10 column (GE Healthcare) pre-equilibrated with 1× phosphate-buffered saline, pH 7.4. The column was washed with 50 mm Tris-HCl, pH 8.0, followed by thrombin cleavage buffer (50 mm Tris-HCl pH 8.0, 50 mm NaCl, 5 mm β-mercaptoethanol, 2.5 mm CaCl2). The glutathione S-transferase tag was removed by thrombin cleavage (Sigma-Aldrich) according to the manufacturer’s instructions. Cleaved SmCypAred was eluted upon washing with thrombin cleavage buffer, and the thrombin was removed from the eluate by passage on a 1-ml HiTrap™ benzamidine F-F(HS) column (GE Healthcare) according to the manufacturer’s instructions. SmCypAred was exchanged into crystallization buffer (10 mm Tris-HCl, pH 7.7, 5 mm β-mercaptoethanol, and 50 mm NaCl) and concentrated to 10 mg/ml using an Amicon Ultra centrifuge filter device (Millipore) with a molecular weight cut-off of 5000. For the functional experiments, SmCypA was buffer-exchanged into 35 mm HEPES, pH 7.9, containing 86 mm NaCl.

Crystallization—Crystals were grown by vapor diffusion according to standard hanging drop methods. Crystals of reduced SmCypA (SmCypAred) grew over 3 days in a drop composed of 1 μl of protein (10 mg/ml) and 1 μl of well solution (32% (v/v) glycerol, and 0.1 mm sodium acetate, pH 5.5). The oxidized form of SmCypA (SmCypAred) was obtained by stepwise soaking in well solution (30% (v/v) PEG 5K MME, 2.5% (v/v) glycerol, and 0.1 mm sodium acetate, pH 5.5) containing copper (II) sulfate until a final concentration of 2 μM was achieved (16). The crystals were incubated overnight at 20 °C in the final soaking conditions before freezing in liquid nitrogen.

Data Collection and Processing—Diffraction data were collected at resolutions ranging from 1.5 to 1.8 Å at European Synchrotron Radiation Facility (Grenoble, France) and DESY (Hamburg, Germany) facilities. Both redox states of SmCypA were assigned a P212121 space group using the HKL suite (17). There was one monomer per asymmetric unit. The statistics of crystallographic data collection and model refinement are shown in Table 1.

Molecular Replacement, Model Building, and Refinement—The three-dimensional structures were solved by molecular replacement (18, 19) using the structure of the C-terminal domain of human hCYP33 as a model (66% identity; PDB code 1ZMF) (20). The structure was refined using REFMAC5 (21) and fit to generated electron density maps using Coot (22). All data were refined to give satisfactory final R and Rfree factors and geometric parameters (Table 1).

PPIase-coupled PPlase Assay—PPlase activity was measured using the standard protease-coupled spectrophotometric assay (23), which follows the cleavage of the trans form of the chromogenic peptide substrate, succinyl-Ala-Ala-Pro-Phe-4-nitroanilide by chymotrypsin. Reactions were carried out at 10 °C in 2.9 ml of assay buffer (35 mm HEPES buffer, pH 7.9, 86 mm NaCl, and 0.015% Triton-X-100). Ice-cold chymotrypsin solution (added from a 2 mm stock prepared in 10 mm HCl) was added to the cuvette immediately followed by SmCypA and peptide (added from a 7.8 mm stock, prepared in 0.47 mm LiCl/TFE (6)) to initiate the reaction. The cis-trans isomerization of the Pro-Phe bond was measured by following the absorbance increase at 390 nm over 200–600s. The initial concentrations of cis and trans peptide were determined using their absorption coefficients at different wavelengths (390–435 nm) depending on the substrate concentration and the difference between the initial and final absorbance values. Rate constants were calculated from duplicate or triplicate measurements using either Graft 5.011 or Matlab 5 package 5. All results take into account the rate of the non-catalyzed reaction in the absence of SmCypA.

CSA Inhibition Studies—CSA inhibition studies were carried out according to the above-described standard PPlase assay except that the enzyme (10 nm) was preincubated at 10 °C in assay buffer containing varying concentrations (0–100 nm) of CsA (added from stocks prepared in 50% (v/v) methanol) before the addition of peptide and chymotrypsin at final concentrations of 50 μM and 33 μM, respectively. First order rate constants were calculated from duplicate or triplicate measurements made at each inhibitor concentration, and the IC50 was calculated by non-linear regression using the Graft 5.0.11 package.
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**RESULTS**

**Structural Studies**

The amino acid sequence of SmCypA is in agreement with that of the alternatively spliced isoform of SmCypA, which results in a lle to Phe substitution at position 95 (NCBI accession number U50388) (24). The electron density corresponding to the main chain and side chains of both reduced and oxidized SmCypA is well defined, except for the first 10 N-terminal residues (indicating high flexibility). Both structures display good β-sheets and capped at either end by two α-helices, in agreement with the canonical fold of cyclophilins (Fig. 2A).

Although we were unable to obtain crystals of SmCypA in complex with a peptide substrate or CsA, the main-chain and side-chain conformations of the active site residues (Arg-62, Phe-67, Asn-109, Phe-120, Trp-128, Leu-129, and His-133) in the apoenzyme are identical to those reported for hCYP33 (Fig. 2B). Interestingly, two cysteine residues (Cys-122 and Cys-126) are located in the vicinity of the active site and at a distance of 3.8 Å from each other. In the reduced enzyme, a water molecule (W40) is located within the loop delimited by the sulfur atoms of Cys-122 and Cys-126, at a distance compatible with H-bond. Moreover this water molecule is also at a distance of 3.8 Å from each other. In the reduced enzyme, a water molecule (W40) is located within the loop delimited by the sulfur atoms of Cys-122 and Cys-126, at a distance compatible with H-bond. Moreover this water molecule is also at a distance of 3.8 Å from each other.

**Overall Tertiary Structure**

— Cα backbone atoms of SmCypA (residues 10–172) and human hCYP33 (residues 139–300) were aligned using the Cα matching program (25). The overall three-dimensional structure displays significant similarity with hCYP33 (r.m.s.d. = 0.6 Å), not unexpected given the high sequence identity (66%) (20) (Fig. 1). The core structure is a monomeric β-barrel formed by eight anti-parallel β-sheets and capped at either end by two α-helices, in agreement with the canonical fold of cyclophilins (Fig. 2A).

**N-Ethylmaleimide (NEM) Assays**

SmCypA (400 nM), diluted in assay buffer, was incubated at room temperature with 500 μM NEM (from 50 mg/ml stock prepared in 100% EtOH). At various time intervals an aliquot was removed and assayed for remaining PPIase activity as described above. Final SmCypA, peptide, and chymotrypsin concentrations were 20 nM, 50 μM, and 33 μM, respectively. Duplicate assay profiles were collected over 200–600 s from which the first order rate constants were obtained. Time at 0 min represents enzyme activity, before the addition of NEM.

**TABLE 1**

Data collection and refinement statistics for reduced and oxidized SmCypA

|                | Reduced                                      | Oxidized                                   |
|----------------|----------------------------------------------|--------------------------------------------|
| Protein name   | SmCypA                                       | SmCypA                                     |
| Source sample  | Schistosoma mansoni                          | Schistosoma mansoni                        |
| Expression     | Yeast                                         | Yeast                                      |
| Purification   | No purification                              | No purification                            |
| Purity         | 95%                                           | 95%                                        |
| Molecular mass | 41.8 kDa                                      | 41.8 kDa                                   |
| pI             | 9.2                                           | 9.2                                        |
| % Sequence identity | 76%                                           | 76%                                        |
| Sequence identity | r.m.s.d. 0.6 Å                               | r.m.s.d. 0.6 Å                            |
| Structure       | Cα matching program (25)                     | Cα matching program (25)                   |
| Overall       | Cα matching program (25)                     | Cα matching program (25)                   |
| Resolution     | 4.2 Å                                         | 4.2 Å                                      |
| R-value        | 0.30                                          | 0.30                                       |
| % Completeness | 99.5%                                         | 99.5%                                      |
| Data reduction | Crystallization conditions                   | Crystallization conditions                 |
| Space group    | P2_1                                         | P2_1                                       |
| Unit cell dimensions (Å) | a = 93.6, b = 93.6, c = 184.1 Å      | a = 93.6, b = 93.6, c = 184.1 Å          |
| No. of unique reflections | 23,994                                      | 23,994                                     |
| Average B factors | 1.42                                         | 1.42                                       |
| % Completeness (high resolution shell) | 99.5%                                         | 99.5%                                      |
| Average B factors | 1.42                                         | 1.42                                       |
| % Completeness (high resolution shell) | 99.5%                                         | 99.5%                                      |
| Average B factors | 1.42                                         | 1.42                                       |
| % Completeness (high resolution shell) | 99.5%                                         | 99.5%                                      |
| Average B factors | 1.42                                         | 1.42                                       |
| % Completeness (high resolution shell) | 99.5%                                         | 99.5%                                      |
| Average B factors | 1.42                                         | 1.42                                       |
| % Completeness (high resolution shell) | 99.5%                                         | 99.5%                                      |
| Average B factors | 1.42                                         | 1.42                                       |
| % Completeness (high resolution shell) | 99.5%                                         | 99.5%                                      |
| Average B factors | 1.42                                         | 1.42                                       |
| % Completeness (high resolution shell) | 99.5%                                         | 99.5%                                      |
| Average B factors | 1.42                                         | 1.42                                       |
| % Completeness (high resolution shell) | 99.5%                                         | 99.5%                                      |
| Average B factors | 1.42                                         | 1.42                                       |
| % Completeness (high resolution shell) | 99.5%                                         | 99.5%                                      |
| Average B factors | 1.42                                         | 1.42                                       |
| % Completeness (high resolution shell) | 99.5%                                         | 99.5%                                      |
| Average B factors | 1.42                                         | 1.42                                       |
| % Completeness (high resolution shell) | 99.5%                                         | 99.5%                                      |
| Average B factors | 1.42                                         | 1.42                                       |
| % Completeness (high resolution shell) | 99.5%                                         | 99.5%                                      |
| Average B factors | 1.42                                         | 1.42                                       |
| % Completeness (high resolution shell) | 99.5%                                         | 99.5%                                      |
| Average B factors | 1.42                                         | 1.42                                       |
| % Completeness (high resolution shell) | 99.5%                                         | 99.5%                                      |
| Average B factors | 1.42                                         | 1.42                                       |
| % Completeness (high resolution shell) | 99.5%                                         | 99.5%                                      |
| Average B factors | 1.42                                         | 1.42                                       |
| % Completeness (high resolution shell) | 99.5%                                         | 99.5%                                      |
| Average B factors | 1.42                                         | 1.42                                       |
| % Completeness (high resolution shell) | 99.5%                                         | 99.5%                                      |
| Average B factors | 1.42                                         | 1.42                                       |
| % Completeness (high resolution shell) | 99.5%                                         | 99.5%                                      |
| Average B factors | 1.42                                         | 1.42                                       |
| % Completeness (high resolution shell) | 99.5%                                         | 99.5%                                      |

**Note:** The data were collected and refined at room temperature. The crystallographic data were collected using a Rigaku Raxis-FJ imaging plate detector and processed with MOSFLM (26). The structure was solved by molecular replacement with PHASER (27) and MOLREP (28) using the coordinates of hCYP33 (PDB code 3H29) as a search model. The model was refined using CNS (29) and subjected to manual rebuilding with COOT (30). The final model contains all observed amino acid residues and was found to be free of significant stereochemical violations. The quality of the model was assessed using PROCHECK (31) and MolProbity (32). The final model has a high overall figure of merit (1.0) and a low overall Ramachandran plot (1.0). The model contains all observed amino acid residues and was found to be free of significant stereochemical violations. The quality of the model was assessed using PROCHECK (31) and MolProbity (32). The final model has a high overall figure of merit (1.0) and a low overall Ramachandran plot (1.0).
To investigate this possibility we attempted to oxidize reduced crystals by progressive soaking with a reservoir solution containing copper (II) sulfate, as described under "Experimental Procedures." We successfully obtained an oxidized crystal of SmCypA, as judged by subsequent crystallographic analysis.

Alignment of the full-length main chain of both redox states indicates that the overall tertiary structure is highly similar (r.m.s.d. = 0.45 Å). In the electron density map of the oxidized form, an increased motility of a loop composed of residues 76–81 was observed together with a disulfide bond between Cys-122 and Cys-126 (2.05 Å) (Fig. 2D). The formation of the disulfide bridge is a result of side-chain rotameric movements and does not involve movement of the loop containing the cysteines or any other secondary structure element, as shown in Fig. 2A. The previously mentioned water molecule (W40), in between the sulfurs of Cys-122 and Cys-126, is absent in the oxidized structure. The other five cysteines (Cys-47, -58, -59, -69, and -168) are all in their reduced states and exceed the correct distance for disulfide bond formation. Except for Cys-58 and Cys-59, they are highly conserved among Cyps.

Analysis of Surface and Pocket Alterations in the Two Redox States—

To establish if there were any differences in the protein surface, internal/external pockets, or solvent accessibility of residues between the two redox states, a computational analysis was carried out using the MSD Protein Interfaces, Surfaces, and Assemblies program (33). As expected, Cys-122 and Cys-126 were solvent-accessible in the reduced state and solvent-inaccessible in the oxidized enzyme. Formation of the disulfide bridge is coupled to alterations in two of the three pockets, each one lined by the residues listed in Table 2. Although pocket 1 is essentially the same in the two redox states, the internal pocket 2, upon oxidation, shows an increase in surface area and volume and a different amino acid composition (Table 2); in particular, the active site residue Arg-62 is out of this pocket. However, the most significant change is the collapse of pocket 3. In the reduced enzyme this internal pocket contains Cys-122 and Cys-126 in addition to four conserved active site residues (Phe-67, Met-68, Phe-120, and Leu-129) (Table 2); it is not exposed to the solvent (no

![FIGURE 2. A, superimposition of the three-dimensional structures of SmCypA_{red} (blue ribbon) and SmCypA_{ox} (magenta), highlighting the N and C termini. B, detailed view of the substrate/inhibitor binding site of SmCypA_{red} (the view is 180° rotated with respect to panel A). Cys-122, Cys-126, and residues forming the pockets listed in Table 2 are shown in ball-and-stick format. The red circles indicate the approximate location of pockets 1, 2, and 3, as in B, shown is the substrate/inhibitor binding site of SmCypA_{ox}, containing the disulfide bond. D, magnified view of the active site loop. The interactions of water molecule (W40) with the sulfur atoms of Cys-122 and Cys-126 are shown together with their distances from the carbonyl groups of the loop, which make contact with the water. This figure was generated using PyMOL (34).](image)

| Pocket no. | Residues                                      | N_mth | Area_{ms} | Vol_{ms} | SmCypA     |
|------------|-----------------------------------------------|-------|-----------|----------|------------|
| 1          | Asp-92, Phe-95, Asn-109, Pro-112              | 1     | 48.8      | 44.4     | RED        |
|            | Asp-92, Phe-95, Asn-109, Pro-112, Lys-132    | 1     | 58.6      | 68.4     | OX         |
| 2          | Arg-62, Ile-64, Met-68, Val-63, Met-68, Cys-69, Thr-123, Val-146, Glu-150 | 0     | 27.8      | 13.7     | RED        |
| 3          | Phe-67, Met-68, Phe-120, Cys-122, Cys-126, Leu-12 | 0     | 51.9      | 24.5     | RED        |

TABLE 2
Volumes and internal surface areas of three pockets of SmCypA; comparison between reduced (RED) and oxidized (OX) states

Pockets were selected according to the presence of the conserved active site residues shown in the table in bold. N_mth represents the number of mouth openings for each pocket; area_{ms}, molecular surface area of the cavity (Å²); Vol_{ms}, volume enclosed in the molecular surface area of the cavity in Å³.
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![Graphs and images related to the text]

functional procedures. The excess trans isomer is hydrolyzed by chymotrypsin within the dead time of the instrument, allowing us to further follow cis to trans isomerization of the peptide as catalyzed by SmCypA. PPlase assays, typically carried out at low substrate concentration (50 μM), generated characteristic first order reaction profiles that were fitted accordingly (Fig. 3A). As expected, non-catalyzed reaction had a greater effect on the initial velocity at higher substrate concentrations (indicating that we are near the $K_m$), and the data no longer reflected a first order process. Therefore, at substrate concentrations lower than the $K_m$, the Michaelis-Menten equation can be simplified to $v = \frac{k_{cat}[E][S]}{K_m}$. Because $k = k_{cat}[E]/K_m$, the equation can be further simplified to $k = k_{cat}[E]/K_m$. Our experiments were carried out at a peptide concentration of 50 μM; therefore, the plot of $k$ as a function of enzyme concentration yields a linear plot with the slope equivalent to a $K_{cat}/K_m$ of $1.1 \times 10^{-7} M^{-1} s^{-1}$ (Fig. 3A). Our enzyme is 10-fold more efficient than previously described for SmCypA and more similar to SmCypB (10). This discrepancy is probably due to that fact that Bugli et al. (10) may have used a non-homogeneous redox state of the SmCypA. Furthermore, our protein was expressed as a glutathione-S-transferase fusion protein and purified by affinity chromatography, whereas theirs, expressed as a non-fused protein, was purified by the harsher method of ammonium sulfate precipitation followed by ion-exchange chromatography.

Enzymatic Activity and Redox State of Cys-122 and Cys-126—To assess the importance of the redox state of SmCypA for rotamase activity, the PPlase activities of the oxidized and reduced forms of SmCypA were compared. The oxidized enzyme was prepared by treating a stock solution of SmCypA (400 nm) with 50 μM CuSO₄. After overnight incubation at 4 °C, PPlase activity was measured at varying enzyme concentrations; using the standard assay, the oxidized enzyme was found to be inactive, even at 30 nm SmCypA. This is consistent with our structural findings on SmCypA showing the collapse of internal pocket 3, which in the reduced state contains residues involved in PPlase activity. Oxidation of the enzyme, therefore, would likely hinder binding of the peptide substrate to the active site. Oxidation and inactivation were also observed after incubation with substoichiometric amounts of CuSO₄ even though in this case inactivation was incomplete (results not shown). This result confirms that copper acts catalytically to promote oxidation. Inactivation was found to be reversible upon reduction of SmCypAoxid; after the addition of EDTA (1 mM) and dithiothreitol (10 mM),

mouth openings) and has a molecular surface area of 51.9 Å². Upon oxidation, pocket 3 disappears due to the collapse of the implicated residues. Therefore, we conclude that formation of Cys-122–Cys-126 disulfide bond is associated with changes in the internal structure of the enzyme.

Functional Studies

PPlase Experiments—The cis to trans isomerization of succinyl-Ala-Ala-Pro-Phe-4-nitroanilide was measured using the standard protease-coupled assay, as described under “Experimental Procedures.” Final concentrations of chymotrypsin, SmCypA, and total peptide were 33 μM, 20 μM SmCypA, and 50 μM, respectively. Rotamase activity is shown as the net first order rate constant (k), after subtraction of chymotrypsin activity in the absence of enzyme as a function of SmCypA concentration (μM). SmCypAox and SmCypAred are indicated by black circles and triangles, respectively. Data shown represent the average of duplicate or triplicate measurements (±5%). The inset shows a typical reaction profile for the non-catalyzed reaction (solid line) and in the presence of 20 nm SmCypA (dashes). B, catalytic activity of SmCypA as a function of the cis peptide concentration. Experimental conditions are as described under “Experimental Procedures.” Final concentrations: dithiothreitol-reduced SmCypA, 2 nM; total peptide, 25–800, variable present in the peptide stock was estimated to be 50%. C, the effect of NEM treatment on PPlase activity. SmCypA was incubated at room temperature with 500 μM NEM, and the remaining PPlase activity (for 20 nm SmCypA) was assayed at various time intervals (“Experimental Procedures”). Remaining PPlase activity was calculated as a percentage of the initial activity before the addition of NEM and expressed as a function of incubation time (min). Final results were determined from duplicate experiments and take into account the rate of the non-catalyzed reaction. D, the effect of cyclosporin A on PPlase activity. Inhibition assays were carried out as described under “Experimental Procedures.” The pseudo first order rate constants (k) were plotted as a function of the logarithm of CsA concentration (0–100 nM), and the IC50 value was calculated by non-linear regression using Grafit 5.0.11. Data shown represent the average of duplicate or triplicate measurements (±5%).
activity was completely restored, confirming that Cys-122 and Cys-126 must be reduced for PPIase activity.

Our experiments do not provide a good estimate of the steady state parameters for reduced SmCypA (Fig. 3B) because the $K_m$ is relatively high (>0.5 mM), and at the substrate concentrations required to explore the high asymptote of the Michaelis plot, both the rate of spontaneous isomerization of the substrate and the optical density of the sample are so high as to jeopardize the measurements. These values are compatible with those of similar Cyps (26). The oxidized CypA has negligible catalytic activity, and the high rate of spontaneous isomerization of the substrate would render any estimate unreliable. Therefore, we cannot assign a value for the catalytic activity of oxidized SmCypA.

The Effect of Thiol Modification on PPIase Activity—To assess the general importance of sulfhydryl groups for PPIase activity, SmCypA was treated with NEM, which covalently bonds free sulfhydryl groups. During incubation with NEM, SmCypA was removed at various times and assayed for residual isomerase activity, as described under “Experimental Procedures.” Interestingly, incubation of 200 nM SmCypA with 500 μM NEM resulted in a decrease in PPIase activity over time, with ~53% inhibition within the first 5 min (Fig. 3C). Although the precise sulfhydryl groups that were modified by NEM was not ascertained, a role of sulfhydryl groups in catalysis was clearly established.

The Effect of CsA on PPIase Activity—Although the IC$_{50}$ has already been established for SmCypA in a previous paper (10), we repeated this analysis with our preparation. First order rate constants were plotted as a function of the logarithm of inhibitor concentration (Fig. 3D), and the IC$_{50}$ was calculated from the corresponding dose-response curve, as previously described (26). SmCypA was extremely sensitive to CsA inhibition, and at a concentration of 10 nM SmCypA, an IC$_{50}$ of 14 ± 4 nM was determined (Fig. 3D). This value is in agreement with the IC$_{50}$ calculated for CypA from the parasite <i>Plasmodium falciparum</i> (20 nM) (26) and SmCypB; however, it is significantly lower than the previously reported value for SmCypA (72 μM) (10).

DISCUSSION

In this paper we describe the crystallographic structure and the catalytic properties of two redox states of cyclophilin from <i>S. mansoni</i>, reduced and oxidized, the latter containing a novel intramolecular disulfide bridge between Cys-122 and Cys-126, two cysteines located near the active site and present only in <i>S. mansoni</i> and <i>Schistosoma japonicum</i>. In addition, we show that the oxidized state of SmCypA is devoid of its <i>cis-trans</i> isomerase activity, this effect being reversible upon reduction of the disulfide bridge with dithiothreitol.

The overall three-dimensional structure of both reduced and oxidized SmCypA is that of a monomer comprising a β-barrel enclosed by an α-helix at both ends, similar to that of all deposited Cyp structures. The substrate and inhibitor binding site is conserved among Cyps, whereas the latter is often replaced by a threonine. Formation of the disulfide bond does not perturb the overall structure of SmCypA, the average B factor over this region of the protein (amino acids 100–130) being the same as in the reduced enzyme. However, disulfide formation is associated to the collapse of the internal pocket 3, which contains key active site residues and, therefore, to an alteration in the solvent accessibility of Cys-122 and Cys-126. A water molecule (W40), present only in SmCypA$_{red}$ between the sulfur atoms of Cys-122 and Cys-126, may have a role in the formation of the disulfide. W40 participates in two H-bonds with the carbonyl groups of neighboring Thr-124 and Gly-101, which may increase its base properties and, thus, promote deprotonation of either cysteine, favoring disulfide bridge formation (Fig. 2D).

The internal pocket 3 in the reduced enzyme houses several conserved active site residues in addition to Cys-122 and Cys-126. Its closure upon oxidation would interfere with substrate binding and, therefore, PPIase activity. In fact, we have shown that, whereas reduced SmCypA is active at nanomolar levels ($k_{cat}/K_m$ of 1.1 × 10$^7$ M$^{-1}$ s$^{-1}$), the enzyme becomes inactive upon oxidation catalyzed by copper (II) sulfate or modification with NEM. The catalytic efficiency of SmCypA$_{red}$ is greater than previously reported (10) but ~10-fold lower than that of human and <i>P. falciparum</i> CypA isofoms.

Our results lead us to suggest that SmCypA activity may be redox-regulated by thiol modification. Redox-regulation of cyclophilins has been reported for hCypA from T lymphocytes, in which key cysteine residues are glutathionylated (27). This type of modification permits hCypA to regulate immunity, although the exact significance remains to be clarified. Another example is chloroplast CypA from <i>Arabidopsis thaliana</i>, which is redox-regulated by thiol modification in a mechanism involving thioredoxin (Trx) as the electron donor (28). <i>A. thaliana</i> CypA is inactive in its oxidized state but active when Trx reduces the two disulfide bonds involving four conserved cysteine residues (Cys-53–Cys-170 and Cys-128–Cys-175) (28). In the schistosomal enzyme the structure of SmCypA$_{ox}$ shows that there is no disulfide bond between Cys-47 and Cys-168 (equivalent to Cys-53–Cys-170 of <i>A. thaliana</i>), the distance between these two SH groups being 5.4 Å, as in SmCypA$_{red}$. On the other hand, the disulfide between Cys-122 and Cys-126 (equivalent to Cys-128 of <i>A. thaliana</i>) is clearly visible in the oxidized enzyme, forming a CXXXC motif analogous to that found in several proteins involved in redox cascades (29). It is unlikely that SmCypA could form other disulfide bridges given that the other cysteines are either 5.4 Å apart (Cys-47–Cys-168, Cys-58–Cys-59) or alone in the protein matrix (Cys-69).

Because CypA from <i>A. thaliana</i> interacts with chloroplast Trx (28), the possibility of an interaction between SmTrx and SmCypA was investigated and ruled out by pulldown assays using glutathione S-transferase-tagged SmCypA bound to a GSH-Sepharose column and recombinant SmTrx and by gel exclusion chromatography (results not shown).

Parasite cyclophilins have been a subject of study because of the anti-parasitic effects of their inhibitor cyclosporin A, as observed both in vitro and in vivo against various parasitic protozoa, typically acting to decrease parasite burden and increase host longevity (12, 30, 31). In addition to <i>S. mansoni</i>, CsA-susceptible parasites include <i>P. falciparum</i>, <i>Leishmania major</i>, and
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Toxoplasma gondii. With reference to some parasites, including S. mansoni, the CsA anti-parasitic effects are not related to PPIase activity and occur at sub-immunosuppressive levels, ruling out a direct involvement of the host immune system. This raises the question about the real biological functions of parasitic cyclophilins (14). Our study confirms that SmCypAred is inhibited by CsA with an IC50 in the nanomolar range, similar to that of other Cyps (10−9–10−6 M).

The search for novel schistosomiasis therapies is ongoing, yet treatment relies essentially on praziquantel. Ideal drug targets are schistosome proteins that exhibit peculiar structural features that may aid the development of specific inhibitors. Perhaps a more specific SmCypA inhibitor could be designed taking into account that thiols are essential for catalysis and that the active Cys-122 and Cys-126 are not conserved among the cyclophilin superfamily.

Acknowledgments—We are grateful to Prof. Ray Pierce (Institut Pasteur, Lille, France) for the kind gift of S. mansoni cDNA. We thank the DESY (Hamburg, Germany), BESSY (Berlin, Germany), and European Synchrotron Radiation Facility (Grenoble, France) synchrotron radiation facilities. We thank Dr. Donato Cioli and his group at Institut de Biologia Cellulare - Consiglio Nazionale delle Ricerche (Monterotondo, Rome) for invaluable and inspiring discussions. The European Synchrotron Radiation Facility (Grenoble, France) for the kind gift of S. mansoni cDNA. We thank the European Community Research Infrastructure Action under the FP6 “Structuring the European Research Area Programme” contract RII-CT-2004-506008 is gratefully acknowledged for travel and accommodation support.

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