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Cloning, expression, and purification of human cyclophilin in *Escherichia coli* and assessment of the catalytic role of cysteines by site-directed mutagenesis

(peptidyl-prolyl cis–trans isomerase/rotamase/tetrahedral intermediate/induced fit/catalytic efficiency)

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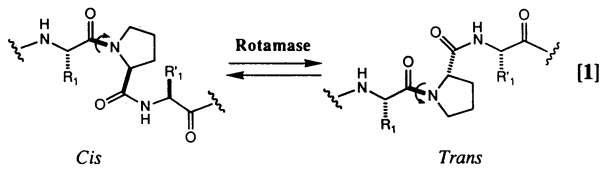
ABSTRACT **The cDNA encoding human cyclophilin from the Jurkat T-cell lymphoma line has been cloned by the expression cassette polymerase chain reaction and sequenced, and an expression vector has been constructed under control of the tac promoter for efficient expression in *Escherichia coli*. Active cyclophilin is produced at up to 40% of soluble cell protein, facilitating a one-column purification to homogeneity. Wild-type cyclophilin was characterized for binding of the potent immunosuppressant agent cyclosporin A (K_d = 46 nM) by tryptophan fluorescence enhancement and for inhibition (IC_{50} = 19 nM) of cyclophilin's peptidyl-prolyl cis–trans isomerase (rotamase) activity. With *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide as the substrate, recombinant human cyclophilin has a high catalytic efficiency; k_{cat}/K_m is 1.4 × 10^7 $M^{-1}\cdot s^{-1}$ at 10°C. To test the prior suggestion that a cysteine residue may be essential for catalysis and immunosuppressant binding, the four cysteines at positions 52, 62, 115, and 161 were mutated individually to alanine and the purified mutant proteins were shown to retain full affinity for cyclosporin A and equivalent catalytic efficiency as a rotamase. Clearly the cysteines play no essential role in catalysis or cyclosporin A binding. These results rule out the recently proposed mechanism [Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T. & Schmid, F. X. (1989) *Nature (London)* 337, 476–478)] involving the formation of tetrahedral hemithioorthoamide. Whereas mechanisms that embody other tetrahedral intermediates may be operative, an alternative mechanism is considered that involves distortion of bound substrate with a twisted (90°) peptidyl-prolyl amide bond.**

The protein cyclophilin (CyP) was identified as a specific high-affinity binding protein for the immunosuppressant agent cyclosporin A (CsA) (1). Because of its dramatic effects on decreasing morbidity and increasing survival rates in human transplants (2, 3), there has been much interest in deciphering CsA's molecular mechanism of immunosuppression. Evidence has accumulated on the one hand that T-cell CyP is a primary target for CsA (1, 4) and on the other hand that the key biological step in immunosuppression is the selective blockade of transcriptional activation of T-cell cytokine genes for production of such molecules as interleukin 2, interleukin 3, and granulocyte–macrophage colony-stimulating factor (5, 6) by the inhibition of specific transcriptional activators such as nuclear factor of activated T cells (NF-AT) (7). An intriguing clue as to how CyP and the FK506-binding protein (FKBP) (8, 9) may affect signal transduction for T-cell activation came with the observation that when a porcine enzyme, peptidyl-prolyl cis–trans isomerase (referred to hereafter as rotamase), which catalyzes the



[1]

peptide bond rotation as shown in Eq. 1, was sequenced it was identical to CyP (10, 11). Thus the hypothesis has been forwarded that CsA and FK506 may function as potent immunosuppressants by interfering with the action of CyP and FKBP (8, 9, 11), respectively. For example, they may block the action of these two enzymes as "foldases" of specific protein substrates (e.g., a subset of T-cell transcription factors specific for cytokine gene activation).

To gain insights into the specificity of a protein such as CyP for its immunosuppressant ligand and to characterize the mechanistically obscure rotamase activity, it seemed useful to clone and express the human cDNA in a heterologous host for overproduction and for structure–function studies. In this report we describe the cloning of the human T-cell CyP from the Jurkat lymphoma cell line (12) using the expression cassette polymerase chain reaction (ECPCR) method (13), its expression, overproduction, and purification from *Escherichia coli*, and initial functional characterization of both wild-type protein and its four cysteine to alanine mutants for rotamase enzymatic activity, and ability to bind to, and be inhibited by, CsA.

MATERIALS AND METHODS

Materials. The polymerase chain reaction (PCR) kit was purchased from Perkin–Elmer/Cetus. The Sequenase kit was from United States Biochemical. Restriction enzymes and replicative forms of M13mp18 and M13mp19 were purchased from New England Biolabs. The tetrapeptide substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide and chymotrypsin were purchased from Sigma. The human Jurkat T-cell total RNA·cDNA heteroduplex [total RNA treated with poly(T) primer and reverse transcriptase] was obtained from Robert Standaert of these laboratories. The rat CyP gene cloned into the plasmid pGEM4Z was kindly supplied by M. Harding (Vertex Pharmaceuticals). The expression vector pHN1+ and the *E. coli* host XA90 F′ *lacI*^{Q1} were generous gifts from Gregory Verdine (Harvard University). CsA and its tritium-labeled derivative were gifts from Sandoz Pharmaceutical. The PCR primers used to amplify the different CyP cDNAs

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Abbreviations: CsA, cyclosporin A; PCR and ECPCR, polymerase chain reaction and expression cassette polymerase chain reaction, respectively; CyP and rhCyP, cyclophilin and recombinant human cyclophilin, respectively; S/D sequence, Shine/Delgarno sequence; FKBP, FK506 binding protein.

(designed according to the ECPCR protocol) and primers used for the site-directed mutagenesis were synthesized by Alex Nausbaum (Harvard Medical School). The PCR primers are as follows. [For the 5′ primers, the Shine/Delgarno (S/D) sequences are underlined and the start ATG codons are in bold face; for the 3′ primers, the complementary sequences of the stop codons are in bold face.] Primer 1,5′-AGCCTACAGCGAATTCTTAACCAGGGAGCTGATTATGGTTAACCCCACCGTGTTCTTCGACATT-3′; primer 2, 5′-ATCGCTAATGAATTCTAGGAGGAATACTTAATGGTTAACCCCACCGTGTTCTTCGAC-3′; primer 3, 5′-GCATGCAAGCTTCTGCAG-TCTAGATTATTCGAGTTGTCCACAGTCAGC-3′; primer 4, 5′-GCGAATTCTTTAAGAAGGAGATATACATATGGTCAACCCCACCGTGTTC-3′; primer 5, GCATGCAAGCTTCTGCAGTCTAGATTAGAGTTGTCCACAGTCGGAGATGGT-3′. The primers for site-directed mutagenesis are as follows. (In all four cases, the mutagenesis involved a CA → GC change in the noncoding strand, which are underlined.) Primer m-1, 5′-CTGTGAAAGGCGGAACCCTT-3′; primer m-2, 5′-CCACCCTGAGCCATAAACCC-3′; primer m-3, TTGGCAGTGGCGATGAAAAA-3′, primer m-4, 5′-AGTTGTCCAGCGTCAGCAAT-3′.

PCR Amplification of CyP Genes. Combinations of primers 1-3 and 2-3 were used to clone the human Jurkat CyP gene. A combination of primers 4-5 was used to amplify the rat CyP gene from the plasmid pGEM-4Z. Each PCR mixture contained (in a total of 100 μl) 20 mM Mops (pH 8.0), 5 mM $MgCl_2$, 5 mM sodium citrate, gelatin at 200 μg/ml, all four dNTPs (each at 300 μM), the two primers (each at 1 μM), 0.5 μg of the cDNA (or 50 ng of plasmid DNA), and 2.5 units of *Thermus aquaticus* DNA polymerase. The PCR was carried out in 30 cycles, each cycle was 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C. At the end of the 30th cycle, the samples were cooled to 37°C over a period of 25 min, and each sample was incubated with 0.5 unit of the Klenow fragment of DNA polymerase I at 37°C for 10 min. Subsequently, the aqueous phase was separated and extracted with phenol/chloroform, and the DNA was precipitated with 0.5 vol of 7.5 M ammonium acetate plus 3 vol of ethanol.

DNA Manipulations. The purified PCR fragments were digested with *Eco*RI and *Hin*dIII and cloned into M13mp18 and M13mp19. The nucleotide sequences of each cloned CyP gene and the mutants were analyzed using the dideoxynucleotide-termination method (14) with the Sequenase kit. The Kunkel method (15) was used for site-directed mutageneses of the four cysteines to alanines, respectively, in CyP on their M13 templates. The cloning of the CyP genes, both the wild type and the mutants, from M13 into pHN1+ and transformation into competent *E. coli* XA90 to construct the overexpression strains were as described (16, 17).

Protein Purification. Two buffers were evaluated in the purification of the recombinant human cyclophilin (rhCyP), 20 mM Tris·HCl (pH 7.8) versus 20 mM Tris·HCl (pH 7.8)/5 mM dithiothreitol/5 mM EDTA. Purification with the first buffer gave homogeneous rhCyP after a DEAE-Sepharose CL-6B column but the second buffer required an additional Superose-6 gel filtration column. Since rhCyP purified with either buffer has identical rotamase and CsA binding activities, the simpler procedure with 20 mM Tris·HCl is described.

Preparation of crude cell extracts. A 2-liter culture of *E. coli* XA90/pHNJ was grown to 37°C in LB medium containing ampicillin (100 μg/ml) to an A_{595} of 0.7; isopropyl β-D-thiogalactopyranoside was added to 2 mM and the incubation was continued for an additional 12 hr. The cells were harvested (wet weight, ≈9 g), washed with 20 mM Tris·HCl (pH 7.8), and resuspended in 36 ml of the same buffer. (This, as well as all subsequent steps, were carried out at 4°C.) The cells were lysed by two passages through a French press at 20,000 psi (1 psi = 6.9 kPa). After separation of the cell debris by centrifugation (20,000 × *g*, 25 min), nucleic acids were precipitated by protamine sulfate (0.4% final concentration). Subsequent centrifugation (20,000 × *g*, 20 min) gave a crude cell extract (44 ml), which was subjected to ammonium sulfate fractionation. The 40–60% (wt/vol) ammonium sulfate pellet was collected, resuspended in 40 ml of 20 mM Tris·HCl (pH 7.8), and dialyzed against 2 liters of the same buffer overnight.

DEAE-Sepharose CL-6B chromatography. The dialyzed crude cell extract was loaded onto a DEAE-Sepharose column (1.2 × 22 cm) equilibrated with 200 ml of 20 mM Tris·HCl and material was eluted with the same buffer. SDS/polyacrylamide gel analysis (18) of flow-through fractions showed most fractions contained essentially pure CyP (Fig. 1). These fractions were combined and concentrated in an Amicon ultrafiltration cell (PM10 membrane).

Protein Characterizations. Proteins were analyzed by denaturing SDS/polyacrylamide gel and concentrations were determined by either the Bradford method (19) or UV absorbance at 280 nm after amino acid analysis. (An average of three runs gave A_{280} = 0.44 at 1 mg/ml.) The N-terminal sequence and amino acid composition were determined by W. Lane (Harvard University microchemistry facility). Isoelectric points were estimated on a Pharmacia Phast gel system.

Rotamase activity assay. The assay developed by Fischer *et al.* (20, 21) was used with minor modifications. A typical 1-ml assay mixture consisted of 35 mM Hepes buffer (pH 8.0), 100 μM *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide dissolved in 66% (vol/vol) aqueous dimethyl sulfoxide, 10 μM chymotrypsin, and rotamase at 2.7–27 nM. All components of the assay mixture except for the chymotrypsin were combined and preincubated at 10°C. Chymotrypsin was added and quickly mixed to initiate the reaction, and an absorbance reading at 390 nm was collected every 0.5 sec by a MacIntosh/Perkin–Elmer λ-2 UV spectrophotometer integrated system, which was designed and assembled by R. Spencer (Pfizer Central Research). First-order rate constants (k_{obs}) were derived by nonlinear least squares fitting of the untransformed data to a simple exponential curve. In all cases the rms residuals were <0.001 A_{390}.

IC_{50} determination. The same rotamase assay (using 5 nM rhCyP) as described above was performed except that various amounts of CsA (1–200 nM final concentration, from a 20 mM stock solution in 36% aqueous dimethyl sulfoxide) were added.

K_d of CsA binding. A procedure analogous to that described by Handschumacher *et al.* (1) was used based on the enhancement of tryptophan fluorescence upon binding of CsA to CyP, except that only unlabeled CsA was present. In a typical titration, 300 nM CyP in 5 mM sodium phosphate (pH 7.2) was mixed with increasing amounts of 10 μM CsA dissolved in 5 mM sodium phosphate (pH 7.2) containing 40%

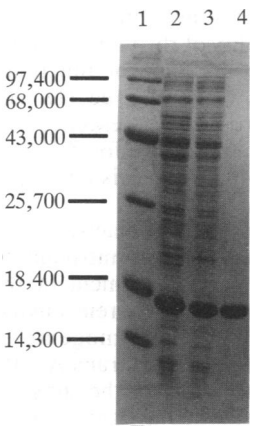

FIG. 1. SDS/polyacrylamide gel (14%) analysis of recombinant human Jurkat CyP/rotamase at various stages of purification. Lanes: 1, molecular weight standard; 2, crude lysate; 3, 40–60% ammonium sulfate pellet; 4, combined DEAE-Sepharose CL-6B fractions.

ethanol (0–700 nM CsA) and the fluorescence was recorded. A plot of the fractional fluorescence change versus the total concentration of CsA gave a sigmoidal curve, from which a K_d can be estimated (see Fig. 2).

RESULTS

ECPCR Cloning of CyP Genes from Human and Rat. To prepare for heterologous expression of eukaryotic CyP genes in *E. coli*, the ECPCR was used to clone the CyP cDNA from a cDNA library based on the sequence information available in the literature (12). Oligonucleotide primers were designed to place a convenient restriction site at each end of the amplified fragment and a prokaryotic-type S/D sequence 6–8 base pairs in front of the anticipated translation start site to set the genes up for expression (13, 22). Products from the PCR incubations were subcloned into M13mp18 and M13mp19 to determine the sequence of the cloned genes. DNA sequencing confirmed successful cloning of the CyP gene from human Jurkat T-cell lymphoma and from rat with identity to the literature sequences (12, 23).

Expression, Overproduction, and Purification of Human CyP. To assess whether efficient expression of the eukaryotic genes could be obtained in *E. coli*, the human Jurkat CyP gene was first cloned into the pHN1+ vector (H. Nash and G. Verdine, personal communication) under control of the tac promoter and expressed in host strain XA90 F′ $lacI^{Q1}$. When primer 1 with a 6-base-pair spacing between the S/D site and the first ATG was used in PCR cloning, the *E. coli* expression was detectable at an estimated 4–5% of soluble cell protein. To optimize expression, this human gene was recloned using primer 2 with an 8-base-pair spacing between the S/D sequence and the translational start site. This 2-base extended interval as well as the alteration of the S/D sequence resulted in a 10-fold increase in expression when inserted into pHN1+ and expressed in *E. coli* strain XA90 such that the recombinant human CyP constitutes ≈40% of the soluble cell protein, as assessed in crude extracts by SDS/PAGE.

At this level of expression the purification of rhCyP became simplified (French press treatment, protamine sulfate precipitation, ammonium sulfate precipitation, and a DEAE-Sepharose CL-6B column). CyP was eluted in the flow-through fractions (it has pI >9.0) and was essentially pure as noted in Fig. 1. Some of the mutant CyPs noted below required additional purification on a Superose 6 column to approach homogeneity. The purified rhCyP has the anticipated molecular mass of 18 kDa. A typical yield from a 2-liter culture is 35 mg of purified rhCyP. N-terminal sequence analysis of the first 14 amino acids confirmed that predicted by the Jurkat gene sequence (12) and the protein from human spleen (23), with 89% retention of the N-terminal methionine in the purified CyP population.

Functional Properties of rhCyP. *CsA binding and apparent K_d by fluorescence excitation.* CyP was isolated in 1984 from bovine thymus and named for its high-affinity binding to CsA (1). Therefore, assessment of the ability of rhCyP to bind this immunosuppressive agent was the first line of functional characterization. CsA binding has been assayed (1) with bovine CyP by enhancement of tryprophan fluorescence with a K_d = 200 nM. Fig. 2 shows a titration curve for rhCyP in this assay with an estimated K_d of 46 nM, consistent with a properly folded protein.

Assay of rotamase activity. The rotamase activity was assayed using *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide. Fischer *et al.* (20, 21) pioneered the development of this substrate and its use in a coupled assay wherein chymotrypsin specifically cleaves off the chromogenic *p*-nitroaniline (monitored at 390 nm) only for the trans Ala-Pro conformer of the substrate. Because <12% of the substrate is in the cis Ala-Pro form, the assay has a signal-to-noise problem and it presents a challenge to quantitative analysis of the rotamase activity.

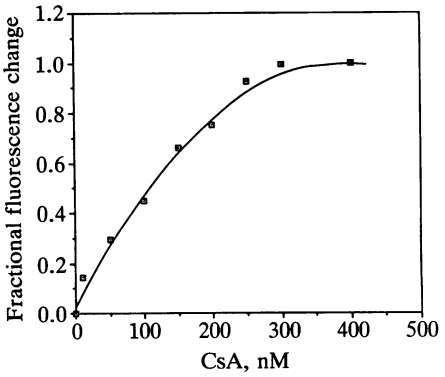

FIG. 2. Titration of CsA binding to CyP by analysis of tryptophan fluorescence enhancement. The CyP concentration was held constant at 300 nM (by Bradford assay) and increasing levels of CsA generated the fluorescence enhancement shown. Given the 1:1 stoichiometry, an apparent K_d can be estimated at a fractional fluorescence change of 0.5. By assuming 50% occupancy, the bound CsA equals free CsA, then the concentration of CsA divided by 2 at 0.5 fluorescence enhancement is the apparent K_d.

Assay of rhCyP with this chromogenic substrate produced data, as shown in Fig. 3*A*, where curve 1 is in the absence of CyP and curve 2 in the presence of 27 nM rhCyP. Variation of the amount of enzyme leads to a family of curves and generates the linear k_{obs} versus CyP concentrations in Fig. 3*B*.

Fisher *et al.* (10) noted that, at the assay concentration of 22 nM substrate, this value of substrate was much less than K_m. Then the Michaelis–Menten velocity equation simplifies to $V = k_{cat}[E_{total}][S]/K_m$. Since $[E_{total}]$ and $[S]$ are known, the k_{obs} values of Fig. 3*B* can be converted to k_{cat}/K_m values. A full kinetic analysis will be detailed elsewhere (R. W. Spencer and W. S. Faraci, personal communication). As shown in Table 1, the wild-type rhCyP displays a k_{cat}/K_m of

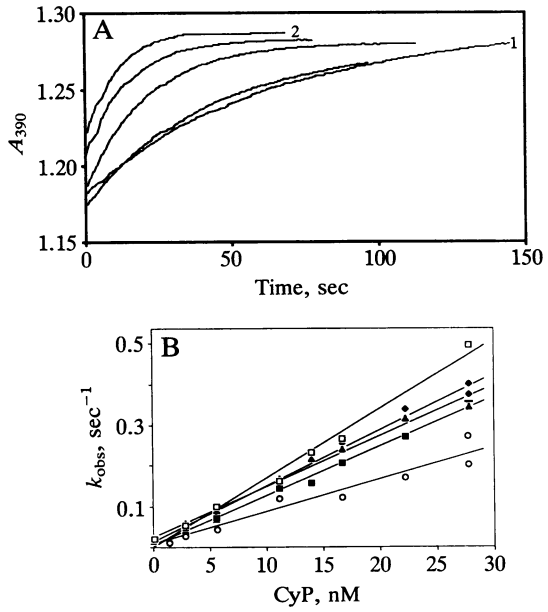

FIG. 3. (*A*) Chymotrypsin-coupled chromogenic assay of rotamase activity with various amounts of the wild-type rhCyP. Curves: 1, no rhCyP; 2, 27 nM rhCyP. (*B*) Variation of observed velocity, k_{obs}, with concentration of wild-type and mutant human CyP/rotamases. ◆, Wild type; ■, Cys-52 → Ala; ▲, Cys-62 → Ala; ○, Cys-115 → Ala; □, Cys-161 → Ala. As indicated in the text, the slope of each line equals k_{cat}/K_m for the corresponding protein as listed in Table 1.

Table 1. Kinetic and binding constants for the wild-type and mutant rhCyP/rotamases

CyP	k_{cat}/K_m ($\times 10^7$), $s^{-1} \cdot M^{-1}$	IC_{50}, nM	K_d, nM
Wild type	1.37	19	46
Cys-52 → Ala	1.21	29	56
Cys-62 → Ala	1.24	25	65
Cys-115 → Ala	1.65*	14	67
Cys-161 → Ala	1.70	28	55

*This Cys-115 → Ala sample was only 50% pure by SDS/PAGE and densitometry analysis and the k_{cat}/K_m value has been corrected for this factor.

1.4×10^7 $M^{-1} \cdot s^{-1}$ at 10°C. While temperature dependence has yet to be performed, if a standard Q_{10} of ≈2 is taken, then at 37°C a value of 7×10^7 $M^{-1} \cdot s^{-1}$ is anticipated. This k_{cat}/K_m, the normal criterion for catalytic efficiency of an enzyme, is very close to the 1×10^8 to 1×10^9 $M^{-1} \cdot s^{-1}$ upper diffusional limit for "kinetically perfect" enzymes (24–26) and suggests that by at least one measure rhCyP is a highly effective catalyst in the cis–trans isomerization of the Ala-Pro bond in this oligopeptide substrate. It will also serve as a benchmark to compare CyPs from other sources and other rotamases such as FKBP, which is specific for a distinct immunosuppressant ligand. With this absolute measure of catalytic activity, we note that, under the particular assay conditions employed, the enzymic/nonenzymic rate is ≈20/1 for acceptable experimental signal to noise. It is unclear what k_{cat}/K_m values will be obtained for other Xaa-Pro cis–trans isomerizations and what effect peptide sequence context and size will have on the catalytic efficiency.

Inhibition of rotamase activity by CsA. Takahashi *et al.* (11) and Fisher *et al.* (10), in describing the identity of porcine rotamase with CyP, characterized CsA as an inhibitor of rotamase activity and obtained K_i values of 2.6 nM (11) and 30 nM (10), respectively. As shown in Fig. 4 and Table 1, the rotamase activity of rhCyP is inhibited by CsA. Since the assay (the concentration of CyP was kept constant at 5 nM while the concentration of CsA ranged from 1 to 200 nM) is not under pure Michaelis–Menten conditions (i.e., $[E] << [S], [I]$), only an IC_{50} rather than a K_i value can be obtained. An IC_{50} value of 19 nM was found for the wild-type rhCyP, in agreement with the previously reported K_i values.

Preparation and Characterization of Cysteine Mutants of rhCyP. Handschumacher *et al.* (1) had shown that CsA binding to bovine CyP was blocked by sulfhydryl reagents and Fischer *et al.* (10) determined that the binding of CsA and the rotamase activity of porcine CyP were inhibited by the mercurial reagent *p*-(hydroxymercuri)benzoate, a modification kinetically protectable by CsA. Of four cysteines modified, the most slowly reacting one was implicated and led

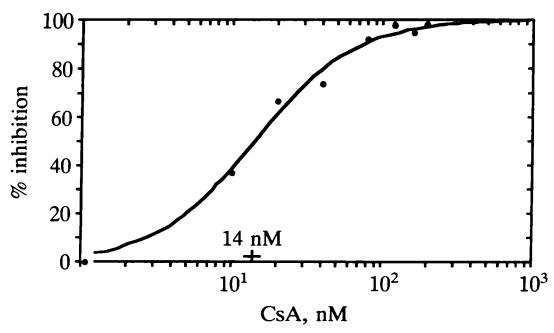

FIG. 4. Concentration dependence of rotamase activity by CsA. The data shown are for the Cys-115 → Ala mutant and are representative of the data for other mutants and wild-type enzyme. The IC_{50} value of 14 nM is indicated by the symbol +.

Fischer *et al.* (10) to predict that "this cysteine is directly involved in the catalytic reaction of peptidyl-prolyl cis–trans isomerase." To this end we decided to assess which, if any, of the four cysteines at positions 52, 62, 115, and 161 might be involved. All four cysteines are conserved between bovine and human CyP protein sequences whereas the rat cDNA sequence predicts a serine at position 52 but a cysteine at position 21, thus maintaining four cysteines. In terms of an initial mechanistic hypothesis of how rotamase might be an Xaa-Pro isomerase (e.g., the cis Ala-Pro link to trans Ala-Pro in the substrate at hand) the idea of a mechanistic variant of a thiol protease, with an active site cysteine as nucleophile, was permissible given the homology in CyP residues 50–57, Gly-Ser-Cys-Phe-Xaa-Xaa-Ile-Ile, to the papain active site sequence at residues 23–32, Gly-Ser-Cys-Trp-$(Xaa)_4$-Val-Val, in which Cys-25 is the catalytic nucleophile (27).

Thus, we individually mutated the four cysteines in the human CyP gene to alanine by the Kunkel method (15), expressed, and purified each of the mutant proteins to homogeneity. As the data of Fig. 3*B* and Table 1 reveal, each C → A mutant of rhCyP is fully functional in binding CsA by the tryptophan fluorescence assay and in terms of k_{cat}/K_m catalytic efficiency criteria, with no apparent diminishment in activity relative to wild-type enzyme. Clearly, the mercuration data were misleading with regard to predictive ability about an essential role for cysteine either in immunosuppressive drug binding or for catalytic activity. Rather they seem to suggest that one of the four cysteines in CyP may be very close to the active site and its modification by *p*-(hydroxymercuri)benzoate can cause the inactivation of rotamase activity due to steric hindrance, an electronic effect from the negatively charged carboxylate from *p*-(hydroxymercuri)benzoate, or both.

DISCUSSION

This work reports the cloning by the ECPCR method of a human T-cell CyP gene and the construction of a highly efficient overproducing vector under control of the tac promoter for expression of large amounts of active protein in *E. coli*. By alteration of the whole ribosome binding site, especially the control of the spacing between a prokaryotic S/D sequence and the translational start site (28), the gene was expressible as 40% of the soluble cell protein, enabling a convenient rapid single-column isolation procedure to obtain large quantities of pure protein. These are useful attributes to a system for exploring structure and function questions in this immunosuppressant target protein, as we have exemplified by the four cysteine mutants described here.

The rhCyP from *E. coli* is functional as a CsA binding protein with an estimated apparent K_d of ≈46 nM, comparable to K_d values of 5–200 nM quoted for CyPs from other species (23). Furthermore, the human CyP is catalytically active in the rotamase chymotrypsin-coupled assay with an N-blocked Ala-Ala-Pro-Phe-*p*-nitroanilide chromogenic substrate. We report here quantitative data for pure CyP in terms of enzyme catalytic efficiency, and the k_{cat}/K_m value of 1.4×10^7 $M^{-1} \cdot s^{-1}$ (10°C) reveals the enzyme to be remarkably proficient as an isomerization or "rotamase" catalyst. As this value approaches the diffusional upper limit for catalysis, one practical conclusion is that most if not all of the human CyP molecules isolated from *E. coli* are in an active form. With partially purified porcine rotamase, Fischer *et al.* (21) had noted a k'_{cat}/K_m of 1.46 liter/mg·min, but the purity of that preparation had not been established and no comments on catalytic efficiency were made. It will be of interest to determine k_{cat}/K_m values for Xaa-Pro linkage cis–trans isomers in protein substrates to assess potential efficiency of CyP/rotamase as a "foldase" for physiological substrates.

As a probe of structural and functional constraints on both immunosuppressant ligand binding and catalytic activity, we

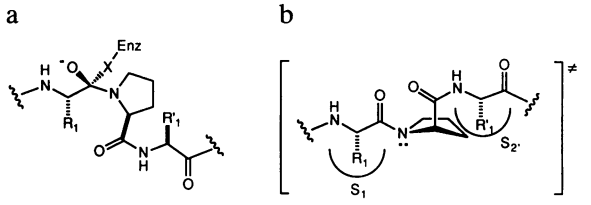

FIG. 5. Two alternative mechanisms for rotamase-catalyzed peptidyl-prolyl cis–trans isomerization as shown in Eq. 1. (*a*) Covalent catalysis in which a nucleophile in the enzyme active site attacks the carbonyl carbon to form a tetrahedral intermediate (X ≠ S). (*b*) Noncovalent catalysis in which the enzyme utilizes its binding energy to selectively stabilize the transition state with a 90° rotation of the amide bond out of planarity (induced fit mechanism).

have used this heterologous expression system to test the prediction (10) that a cysteine residue was crucial for both functions. In fact with all four single cysteine mutants (as well as a double Cys → Ala mutant, data not shown), we have demonstrated that each cysteine is dispensable, despite the strict positional conservation of cysteines shown in bovine and human sequences and three of four in rat sequences. After these experiments were concluded, the yeast CyP sequence was published (29), and there is no conservation of the placement of any of the cysteines between the human and yeast sequences. The yeast CyP is reported to have weak but detectable rotamase activity. The availability of the yeast CyP gene will permit genetic assessment for its role in folding of essential proteins.

While our results clearly rule out a mechanistic route to Xaa-Pro peptide isomerization involving a thiol nucleophile to form a tetrahedral adduct, there remain three other mechanistic classes of proteases, the "serine" proteases (30), metallo proteases (31), and "aspartyl" proteases (32) as possible precedents. CyP is active in solutions containing up to 5 mM EDTA, ruling against a key role for divalent cations in rotamase activity. It remains to be seen if other nucleophiles in the enzyme active site, such as a hydroxyl group from serine or a carboxylate from aspartate or even an enzyme-bound water molecule, may attack the carbonyl carbon to form a tetrahedral intermediate during catalysis (Fig. 5*a*). It is also possible that an alternative mechanism may be operative that involves stabilization of a twisted peptidyl-prolyl amide bond (induced fit mechanism; Fig. 5*b*). This mechanism should be given careful consideration for both CyP and FKBP.

The cloning and expression of a human T-cell CyP gene in *E. coli* and its facile purification in quantity should enable characterization of its specificity for immunosuppressant ligand and recognition features for substrates, from the tetrapeptide anilide chromogenic substrate to such proteins as denatured ribonuclease T1 in refolding assays. The quantitative determination of human CyP catalytic efficiency (k_{cat}/K_m) can be applied to other CsA binding proteins, including the ninaA protein of *Drosophila* (33, 34), the FKBP (8, 9), and other "immunophilins" (e.g., with rapamycin as ligand) that are purified. Although structure–activity relationships for CsA analogs correlate between affinity of binding to CyP and *in vivo* immunosuppressant potency (1), thereby implicating CyP as a candidate target protein, the relevance of the rotamase activity as a biological endpoint and the nature of any protein substrates of CyP involved in signal transduction, such as tyrosine kinases, ion channels, or T-cell-specific transcription factors (9), remains to be determined. It is essential to determine if CyPs and also FKBP act physiologically as protein "foldases" in tissue-specific gene activation readouts known to be the downstream biological effects of such immunosuppressants as CsA and FK506.

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