Interactions of Cyclophilin with the Mitochondrial Inner Membrane and Regulation of the Permeability Transition Pore, a Cyclosporin A-sensitive Channel*

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Mammalian mitochondria possess an inner membrane channel, the permeability transition pore (MTP), which can be inhibited by nanomolar concentrations of cyclosporin (CS) A. The molecular basis for MTP inhibition by CSA remains unclear. Mitochondria also possess a matrix cyclophilin (CyP) with a unique N-terminal sequence (CyP-M). To test the hypothesis that it interacts with the MTP, we have studied the interactions of CyP-M with intact liver mitochondria by Western blotting with a specific antibody against its unique N terminus. Although sonication in isotonic sucrose at pH 7.4 releases a large proportion of CyP-M, a sizeable CyP-M fraction sediments with submitochondrial particles at 150,000 × g. We show that the interactions of this CyP-M pool with submitochondrial particles are disrupted (i) by the addition of CSA, which inhibits the pore, but not of CSH, which does not, and (ii) by acidic pH condition, which also leads to selective inhibition of the MTP; furthermore, we show that the effect of acidic pH on CyP-M binding is prevented by diethylpyrocarbonate, which fully prevents the inhibitory effect of H⁺ on the MTP (Nicolli, A., Petronilli, V., and Bernardi, P. (1993) Biochemistry 32, 4461–4465). These data suggest that CyP-M binding is involved in opening of the MTP and that pore inhibition by CSA and protons may be due to unbinding of CyP-M from its putative binding site on the MTP. A role for CyP-M in MTP regulation is also supported by a study with a series of CSA derivatives with graded affinity for CyP. We show that with each derivative the potency at inhibition of the peptidylprolyl cis-trans-isomerase activity of CyP-M purified to homogeneity is similar to that displayed at inhibition of MTP opening, relative to that displayed by CSA. Decreased binding to CyP-M (but not CyP-A) and decreased efficiency at MTP inhibition is obtained by substitutions in position 8 while a 4-substituted, nonimmunosuppressive derivative is as effective as the native CSA molecule, indicating that calcineurin is not involved in MTP inhibition by CSA.

Mitochondria from a variety of sources possess a regulated inner membrane channel, the permeability transition pore (MTP).¹ Pore opening is dependent on both the transmembrane potential difference (1) and on matrix pH (2) and is modulated by a variety of effectors acting at multiple sites (for recent reviews, see Refs. 3 and 4). Among pore inhibitors, CSA stands out for its potency (apparent $I_{50}$ is in the submicromolar range) and for its selectivity (no other mitochondrial functions appear to be affected by this drug) (Refs. 3 and 4 and references therein). In fact, it is because of inhibition by CSA (5–7) that the nature of the “permeability transition” (a Ca²⁺-dependent increase of inner membrane permeability to solutes with molecular mass ≤ 1500 Da) was recently recognized as being due to opening of a channel (8–10), as first proposed by Hunter and Haworth in 1979 (11, 12), rather than to a permeability change of the membrane lipid phase (for review, see Ref. 13).

Inappropriate MTP opening is becoming increasingly recognized as a causative event in cell injury by a variety of conditions, including ischemia (14–16). On the other hand, and although a role of the pore in cellular Ca²⁺ homeostasis appears likely (17–19), most questions regarding its physiological function, regulation, and molecular structure await an answer.

A soluble mitochondrial extract displays PPiase activity, which can be inhibited by CSA and CSG but not by CSH or FK506 (20). Since this pattern is shared by the permeability transition, it has been suggested that a mitochondrial CyP mediates CSA inhibition of the pore (20). Although a 20-kDa CyP isoform (CyP-M) with a unique N-terminal sequence has later been isolated from mitochondria (21), its role in the permeability transition is unclear.

A study of radiolabeled CSA binding to mitochondria defined two classes of high affinity CSA-binding sites, suggesting the possible existence of further CS-binding proteins besides CyP-M (22). In keeping with this hypothesis, a 10-kDa protein could be labeled with a photocative CSA derivative in a membrane-associated fraction obtained after sonication of mitochondria, and it was suggested that the labeled protein was part of the membrane CSA receptor on the pore (23).

The question of whether CSA interacts directly with the pore, or rather whether its inhibitory effects on the pore are mediated by CyP-M is a fundamental one. Besides the intrinsic

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1 The abbreviations used are: MTP, mitochondrial permeability transition pore; Cs, cyclosporin; PPiase, peptidylprolyl cis-trans-isomerase; CyP, cyclophilin; DPC, diethylpyrocarbonate; DTT, dithiothreitol; MOPS, 4-morpholinoethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; SMP, submitochondrial particles; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; FKBP, FK506 binding protein; IP₃R, inositol 1,4,5-trisphosphate receptor; SR-CRC, sarcosplenic reticulum calcium release channel.
important for the mechanistic aspects of MTP function, assessing this point has a specific relevance for any strategy aimed at pore isolation. Indeed, if CyP-M is required for pore opening, any protocol designed for its reconstitution must include this protein.

In this study, we have isolated CSA-binding proteins from a subcellular fraction enriched in mitochondria by affinity chromatography on immobilized CSA. We show that CyP-M is the main and probably the only mitochondrial receptor for CSA and address the question of its role in MTP function with two approaches. In the first, we have studied the interactions of CyP-M with intact rat liver mitochondria by Western blotting with a specific antibody against its unique N terminus. We show that CyP-M is a matrix protein that is engaged in interactions with the mitochondrial inner membrane, and that these interactions are partially retained by SMP. Interactions of CyP-M with SMP are disrupted by the addition of CSA and by mildly acidic pH values, conditions that also lead to selective inhibition of the MTP. On the other hand, CyP-M interactions with the inner membrane are unaffected by CSH, which does not inhibit the MTP, while the effects of acidic pH can be prevented by DPC, which also prevents MTP block by H^+ (2).

These observations have been exploited to devise a novel protocol for CyP-M purification to homogeneity. In the second approach, we have then studied a series of CSA derivatives with graded affinity for CyP-A for their inhibition of CyP-M and of MTP. We find that with each derivative the potency at inhibition of the PPIase activity of purified CyP-M, relative to that of CSA, is similar to that displayed at inhibition of MTP opening. Structure-function analysis of CS derivatives reveals that decreased binding to CyP-M (but not CyP-A) and decreased efficiency at MTP inhibition is obtained by substitutions in position 8, while a 4-substituted, nonimmunosuppressive derivative, which does not bind calcineurin, is as effective as the native molecule at inhibition of the MTP and of CyP-M. Taken together, these data (i) suggest that CyP-M binding is involved in MTP function and that pore inhibition by CSA and H^+ may be due to unbinding of CyP-M from its putative binding site on the MTP and (ii) show that inhibition of calcineurin activity is not required for the inhibitory effects of CSA on MTP.

MATERIALS AND METHODS

Preparation of Subcellular Fractions—Rat liver mitochondria were prepared by standard differential centrifugation (24). SMP were prepared as follows. The mitochondrial stock solution was diluted to about 30 mg x m^(-3) in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 0.1 mM EGTA-Tris. 2-ml aliquots were transferred to 6-ml glass vials (Packard Instrument Co, Meriden, CT), and subjected to three cycles of sonication of 3 min each at room temperature, with 15-min intervals on ice in a G112SPIT water bath Sonifier (Laboratory Supplies Co., New York, NY). The samples were centrifuged at 8,000 x g for 10 min to remove unbroken mitochondria, and the resulting supernatants were centrifuged for 1 h at 150,000 g in the rotor 70 Ti of a Beckman Ultracentrifuge. The supernatants were decanted, the pellets and the tube walls were rinsed twice with 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 0.1 mM EGTA-Tris, and the SMP pellets were resuspended in the desired buffer (1 ml of buffer/30 mg of starting mitochondrial protein), as detailed in the figure legends.

Affinity Chromatography—To identify mitochondrial CSA-binding proteins, isolated mitochondria (250 mg of protein) were extracted with 25 ml of 0.35 M NaCl, 10 mM NaP, pH 7.4, 2% Triton X-100, 0.23 mM phenylmethylsulfonyl fluoride, 0.83 mM benzamidine, and incubated overnight at 4°C with 0.5 ml of a-Ala-(3-amino)-8-CSA Affi-Gel 10 matrix pre-equilibrated with 0.15 M NaCl, 10 mM NaP, pH 7.4, 0.5% Triton X-100, 0.23 mM phenylmethylsulfonyl fluoride, 0.83 mM benzamidine (column buffer). The column was sequentially washed with 10 bed volumes of column buffer, 5 bed volumes of column buffer containing 0.5 M NaCl, and 10 bed volumes of column buffer, followed by elution with 0.42 ml CSA in column buffer and 6 ml urea in NaCl-free column buffer. 1-bed volume fractions were collected, and 15 μl of relevant fractions (as indicated in the legend to Fig. 1) were analyzed by SDS-PAGE and silver staining.

Preparation of CyP-M—CyP-M was purified from SMP isolated as above. Our purification strategy capitalized on the original finding that CyP-M binding to inner membranes can be disrupted by acidic pH values (see Fig. 4). SMP were resuspended in 0.25 M sucrose, 20 mM sodium acetate, pH 5.0, 0.1 mM EGTA-Tris at approximately 10 mg x m^(-1) and centrifuged for 1 h at 150,000 x g in the rotor 70 Ti of a Beckman ultracentrifuge. The supernatants, containing essentially all CyP-M, were adjusted to pH 8.2 with Tris base and further fractionated essentially as described by Connern and Halestrap (21). Briefly, the supernatants (about 20 ml) were applied to a 1 cm x 8 cm Q-Sepharose column (Pharmacia Biotech Inc.). Flow-through, containing all of CyP-M, was loaded onto a 1 cm x 2 cm S-Sepharose column (Pharmacia). After washing with 65 bed volumes of 10 mM Tris-HCl, pH 8.2, 2 mM EDTA-Tris, 0.5 mM DTT and then with the same buffer containing 20 mM NaCl, the S-Sepharose column (which under these conditions retains CyP-M) was finally eluted with 0.1 M NaCl, 10 mM Tris-HCl, pH 9.5, 2 mM EDTA-Tris, 0.5 mM DTT. The fractions containing CyP-M (as assessed by Western blotting with a monospecific antibody, see Fig. 4) were pooled, dialyzed against 10 mM Tris-HCl, pH 8.2, 2 mM EDTA-Tris, 0.5 mM DTT, and then against 50 mM MES, pH 6.0, 2.0 mM EDTA, 0.5 mM DTT, and finally loaded onto a 1 ml column (Pharmacia) and eluted with a 0–250 mM NaCl gradient. CyP-M eluted at approximately 0.1 M NaCl, and was analyzed for purity by SDS-PAGE followed by Coomassie Blue or silver staining and by Western blotting (see Fig. 4).

Antibody Preparation—For preparation of a monospecific antibody, CyP-M was purified by affinity chromatography exactly as described above and concentrated by precipitation with trichloroacetic acid as described previously (25). The 20-kDa CSA-eluted protein of interest was transferred to an Applied Biosystems (Foster City, CA) ProBlott nylon membrane as described under “Analytical Techniques” below, stained with Coomassie Blue according to the manufacturer's instructions (Bio-Rad), and subjected to amino acid sequence analysis with an Applied Biosystems 477A Sequenator equipped with on-line phenylthiodyantoin aminoacid analyzer (model 120A). The N-terminal sequence, determined by Dr. Patrizia Polverino De Laurento of the Centro di Riicerca Interdepartimentale per le Biotechnologie Innovative Biotechnology Center of the University of Padova, was ASDDGARGANS550QPLV. A synthetic peptide with sequence ASDDGARGANS555QPLV and an irrelevant peptide with sequence KVEKIGEYTGYVVKY were prepared by applying an Applied Biosystems 431-A automatic peptide synthesizer and validated by sequencing by Dr. Oriano Marin, CRIBI Biotechnology Center and Department of Biochemistry, University of Padova. After conjugation of the CyP-M peptide to maleimide-activated keyhole limpet hemocyanin (Pierce), the peptide conjugate was subcutaneously injected into New Zealand rabbits at multiple sites in complete Freund’s adjuvant). After 3 weeks, and then at three 4-week intervals, the rabbits were boosted with 1 mg of peptide conjugate in incomplete Freund’s adjuvant, and the appearance of specific antibodies monitored by Western blotting of either affinity-purified CyP-M or of an ASDDGARGANS555QPLV conjugate to bovine serum albumin.

Analytical Techniques—SDS-PAGE was performed according to Laemmli (26) in acrylamide-bisacrylamide slab gels, which were stained with either Coomassie Blue or silver as specified in the figure legends, or transferred to nitrocellulose (0.22-μM pore size, Hoefer, San Francisco, CA) in CAPS-NaOH, pH 11.0, 10% MetOH (overnight at 4°C, 2 mA/cm^2). In the experiment of Fig. 2, the nitrocellulose sheet was stained with Ponceau Red, photographed, destained with dH_2O, and subjected to Western blotting with the monospecific antibody against CyP-M (see Figs. 3, A and B, and 4, two parallel gels were run, one being fixed and stained and the other transferred to nitrocellulose and subjected to Western blotting as above). Using the nitrocellulose blots, a 0.25 μM solution of an alkaline phosphatase-conjugated goat anti-rabbit antibody (Sigma).

Peptide activity was determined with the spectrophotometric method of Fischer et al. (27) and analyzed according to Harrison and Stein (28). The concentration of CyP-M or of recombinant human CyP-A (a generous gift of Dr. Mauro Zurini, Sandz Pharma AG) was adjusted to give an observed first-order rate constant for the catalyzed reaction about 5-fold larger than that of the uncatalyzed reaction.

MTP opening was followed as the rate of absorbance or 90°C light scattering change at 540 nm in 0.20 M sucrose, 10 mM Tris-MOPS, pH 7.4.
mitochondria was subjected to affinity chromatography on D-Ala-(3-amino)-8-CSA. This approach led to the elution of two proteins of 30 and 32 kDa, and their permeability to solutes was measured as described in Ref. 30, and their binding to the affinity matrix remains unclear, and was not investigated further in this paper.

Identical affinity purification protocols were carried out in repeats of this experiment. Elution with 6 M urea occasionally released two proteins of 30 and 32 kDa, and the major CSA-eluted proteins were identified by amino-terminal sequencing. The 30-kDa species had sequence ASDGGARGANSSSQNPLV, which matches that obtained by Connern and Halestrap (21) on a matrix PPiase modelled on the unique rat CyP-M N-terminal sequence. The 30-kDa species remains unknown. Since under no circumstances could these proteins be eluted with CSA, the nature of their binding to the affinity matrix remains unclear, and was not investigated further in this paper.

In the experiments reported in Fig. 3, we have studied the interactions of CyP-M with mitochondria, prepared rabbit antisera against a synthetic peptide modelled on the unique rat CyP-M N-terminal sequence. The experiments depicted in Fig. 2 document the properties of one such antisera. It can be seen that a single 20 kDa band was recognized in Western blots of total mitochondrial proteins (lanes 1 and in the CSA eluate of the D-Ala-(3-amino)-8-CSA affinity matrix (lanes 3). The reaction was specific, in that it was not detected with an identical dilution of preimmune serum (lanes 2), and it was selective for the N terminus of CyP-M in that (i) no reactivity was detected with either CyP-A or CyP-B (lanes 1–3 and 5), but not by an irrelevant KVEK1EGTYGVYK peptide (lanes 4) but not by an irrelevant KVEK1EGTYGVYK peptide (lanes 4). Thus, this antibody is a useful tool to probe interactions of CyP-M with mitochondria.

In the experiments reported in Fig. 3, we have studied the distribution of CyP-M between a membrane and a soluble fraction obtained by ultracentrifugation of a suspension of sonicated mitochondria in a sucrose-based medium (panels A and B). CyP-M was clearly detectable both in the membrane-associated (lanes 3) and in the soluble fractions (lanes 4), showing that a subpopulation of CyP-M molecules interacts with the

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### Table 1: Structure of CSA derivatives

| Derivative          | Substituted amino acid | Substituent amino acid | Modification       |
|---------------------|------------------------|------------------------|--------------------|
| -MeSer-3-CS         | Sarcosine              |                        | Lateral chain amino acid 1 |
| MeVal-4-CS          | N-Methylleucine        |                        | Lateral chain amino acid 3 |
| Val-2MeBmt(6.7-DH)-1-CS | 4Rl-4(E)-2-butenyl-1-threonine (MeBmt) |            | Lateral chain amino acid 4 |
| Nva-2-CS            | l-α-Aminobutyric acid  |                        | Lateral chain amino acid 5 |
| o-MeVal-11-CS (CSH) | N-Methyl-l-valine      |                        | Lateral chain amino acid 6 |
| o-MeSer-3-CS        | N-Methyl-threonine      |                        | Lateral chain amino acid 7 |
| Val-2MeBmt(6.7-DH)-1-CS | 4Rl-4(E)-2-butenyl-1-threonine (MeBmt) |            | Lateral chain amino acid 8 |
| Nva-2-CS            | Norvaline              |                        | Lateral chain amino acid 9 |
| o-MeVal-11-CS (CSH) | N-C-Dansyl-α-lysine    |                        | Lateral chain amino acid 10 |
| o-MeSer-3-CS        | N-Methyl-β-lysine      |                        | Lateral chain amino acid 11 |

7.4, 5 mM succinate-Tris, 1 mM P, 20 mM EGTA-Tris, and 2 μM rotenone. Mitochondria (0.5 mg ml⁻¹) were incubated in thermostatted, magnetically stirred cuvettes (final volume, 2 ml, 25°C). After accumulation of 40 μM Ca²⁺, 0.5 mM EGTA-Tris was added to prevent opening of the Ca²⁺ channel, and MTP opening was then triggered by the addition of 0.2 μM carbonyl cyanide p-trifluoromethoxyphenyl hydrazone (see Ref. 29 for further details and representative traces).

Liposomes reconstituted with mitochondrial proteins were prepared, and their permeability to solutes was measured as described in Ref. 30, and measurements of SMP membrane potential with oxonol VI (Molecular Probes, Eugene, OR) were performed as described previously (31). Identical affinity purification protocols were carried out in

![Fig. 1. Identification of CSA-binding proteins in a mitochondrial preparation by affinity chromatography on D-Ala-(3-amino)-8-CSA.](http://www.jbc.org/content/218/3/1961/F1.large.jpg)
mitochondrial inner membrane, and that this interaction is retained after sonication and despite the large dilution of the matrix following disruption of mitochondria.

The nature of these interactions was investigated further by exposing this SMP preparation to a variety of conditions (Fig. 3, panel C). CyP-M could be released by isotonic NaCl (lanes 2, compare with lanes 1), suggesting that CyP-M membrane interactions are influenced by the ionic strength and demonstrating that all CyP-M is bound to the outer surface of our SMP preparation and accessible to the external milieu. Importantly, a large fraction of CyP-M could be released by CSA, which inhibits the pore, but not by CSH, which does not (lanes 3 and 4, respectively, compare with lanes 1), providing a first indication that CyP-M (un)binding to the inner membrane may be instrumental in MTP operation.

Release of CyP-M could also be detected after exposure of the SMP preparation to an acidic medium (lanes 5, compare with lanes 7), and this effect could be prevented by treatment of SMP with DPC prior to acidification (lanes 6, compare with lanes 7). We have previously shown that matrix acidification is accompanied by MTP closure, due to reversible protonation of histidyl residues, and that the inhibitory effects of matrix H⁺ on the MTP can be prevented by carbethoxylation of critical histidyl residues with DPC (2). Thus, these experiments further support our hypothesis on MTP-CyP-M interactions. It should be noted that a small proportion of CyP-M could be detected in the soluble fraction of the controls (SMP resedimented in sucrose medium at pH 7.4, panel C, lanes 1 and 7), most likely a dilution effect. With no exception, however, exposure to CSA, acidic pH, or isotonic NaCl resulted in an increase of the CyP-M released in the soluble fraction relative to the controls.

To investigate the role of CyP-M interactions in MTP regulation directly, we tried to detect MTP activity in SMP by a variety of methods. These included (i) a study of the permeability properties of liposomes reconstituted with SMP proteins.
soluble fraction was adjusted to pH 8.2, sequentially passed through an anion-and cation-exchange matrix (lanes 1, 3, and 5); elution of S-Sepharose column (peak fraction) (lanes 4); elution of HR 5/5 mono-S column (CyP-M peak fraction eluting at about 0.1 M NaCl) (lanes 5). The position of CyP-M is indicated by the horizontal arrow. For details see “Materials and Methods.”

Fig. 4. Purification of CyP-M. A SMP preparation (corresponding to lanes 3, panels A and B of Fig. 3) was resuspended in isosotic sucrose, pH 5.0, and spun at 150,000 × g for 1 h. The supernatants were brought to pH 8.2, and CyP-M was purified to homogeneity by sequential chromatographic steps on Q-Sepharose, S-Sepharose, and HR 5/5 mono-S HPLC columns as described in detail under “Materials and Methods.” Panel A, SDS-PAGE (15% acrylamide-0.4% bisacrylamide, Coomassie Blue staining) and Panel B, Western blot analysis with anti-CyP-M antibody of 150,000 × g supernatant, pH 5.0 (lanes 1); flow-through of Q-Sepharose column (lanes 2); flow-through of S-Sepharose column (lanes 3); elution of S-Sepharose column (peak fraction) (lanes 4); elution of HR 5/5 mono-S column (CyP-M peak fraction eluting at about 0.1 M NaCl) (lanes 5). The position of CyP-M is indicated by the horizontal arrow. For details see “Materials and Methods.”

In this paper we have (i) characterized CSA-binding proteins associated with isolated mitochondria, showing that the 20-kDa CyP-M is the main and possibly the only mitochondrial CSA binding protein; (ii) studied the interactions of CyP-M with SMP and their modulation by potent inhibitors of the MTP, a CSA-sensitive mitochondrial channel; (iii) devised a new protocol for CyP-M purification; and (iv) studied the inhibitor profile of highly purified CyP-M and of MTP with a series of CSA derivatives with graded affinity for CyP-A. Our results support the idea that CyP-M activity of MTP is essential for channel opening, suggest that MTP inhibition by CSA and protons may be due to CyP-M unbinding from its putative site on the MTP, and show that calcineurin inhibition is not involved in MTP inhibition by CSA.

How Many CSA-Binding Mitochondrial Proteins?—The first relevant result of this paper is reported in Fig. 1. Although three CSA-binding proteins of molecular masses 18, 20, and 22 kDa can be purified by affinity chromatography from a conventional mitochondrial preparation isolated by differential centrifugation, only the 20-kDa species is selectively localized to mitochondria, while the 18- and 22-kDa species, which are both present in a postmitochondrial 150,000 × g particulate fraction (25), are most likely derived from endoplasmic reticulum vesicles, which are associated with the outer mitochondrial membrane. A second point of interest is that no other proteins could be specifically eluted from the CSA affinity matrix (Fig. 1), even after previous removal of CyPs (results not shown). These data suggest that CyP-M is the main and possibly the only mitochondrial receptor for CSA, which makes it the most likely candidate for MTP inhibition by CSA.

Recently, Andreeva and Crompton (23) have exploited the synergistic effects of ADP and CSA at MTP inhibition (36, 37) to identify a 10-kDa protein in a rat liver mitochondrial fraction that could be photolabeled with a tritiated, 8-substituted CSA derivative in the presence of ADP after soluble components had been removed by sonication at pH 8.1 and centrifugation (23). It was proposed that the 10-kDa species is an integral membrane protein, and that it most likely represents the target for CSA on the pore (23). Subsequently, the same group has reported photolabeling also of a 22-kDa protein possessing CSA-sensitive PPIase activity (K, for CSA 5 mM) from rat heart mitochondria, and of a 18-kDa protein from rat liver mitochondria (39) and suggested that the 10-kDa liver species may in fact be a proteolytic product of the 18-kDa protein rather than the CSA membrane receptor (39). In the absence of structural information on these proteins, it is hard
to make predictions about their possible relationships with CyP-M, and with the MTP regulatory mechanism proposed here. However, we note that (i) due to the tight association of the endoplasmic reticulum with mitochondria, both the 22-kDa CyP-B and the 18-kDa CyP-A copurify with the 20-kDa CyP-M in CSA affinity-based protocols (Fig. 1); (ii) the protocol used for membrane preparation by Andreeva and co-workers (sonication at pH 8.1; Refs. 23 and 39) is expected to remove a large fraction of CyP-M (Fig. 3); (iii) the endoplasmic reticulum is expected to retain its integrity in sonication protocols designed for disruption of mitochondria and thus to represent a major component of the vesicular fraction sedimented by ultracentrifugation after sonication; and (iv) the ion-exchange chromatog-

**FIG. 5.** Inhibition of the MTP and of the PPIase activity of CyP-M and CyP-A by CSA and MeVal-4-CS. Determinations of MTP activity (panels A and B) and of the PPIase activity of CyP-M (panels C and D) and of CyP-A (panels E and F) were carried out as described under “Materials and Methods” in the presence of the indicated concentrations of CSA (closed symbols) or MeVal-4-CS (open symbols). CyP-A was the human recombinant species. Vertical bars denote the standard error as obtained from at least three determinations.

| Derivative                  | IC₅₀ MTP (nM) | IC₅₀ CyP-M (nM) | IC₅₀ CyP-A (nM) |
|-----------------------------|---------------|-----------------|-----------------|
| CSA                         | 39 ± 8 (20)   | 6.0 ± 1.7 (6)   | 10 ± 4.0 (3)    |
| o-MeSer-3-CS                | 41 ± 8 (3)    | 2.4 ± 0.5 (3)   | 3 ± 0.8 (3)     |
| MeVal-4-CS                  | 69 ± 4 (3)    | 4.7 ± 1.5 (6)   | 5 ± 1.0 (3)     |
| Val-2-MeBmt(6,7-DH)-1-CS    | 87 ± 9 (3)    | 7.0 ± 0.9 (3)   | 8 ± 3.0 (3)     |
| Nva-2-CS                    | 78 ± 19 (3)   | 6.4 ± 1.3 (3)   | 15 ± 11 (3)     |
| o-Lys(dansyl)-8-CS          | 524 ± 192 (3) | 32.6 ± 9.0 (6)  | 18 ± 4.0 (3)    |
| o-MeVal-11-CS (CSH)         | No inhibition | No inhibition   | No inhibition   |

**TABLE II**

Inhibition of MTP, CyP-M, and CyP-A by CSA and derivatives

The determinations were carried out exactly as described in the legend to Fig. 5. For each derivative, at least triplicates of 5–7 concentrations were used to determine the IC₅₀. The values given are in nM ± S.D., and values in parentheses refer to the total number of titrations performed.

| Derivative                  | IC₅₀R MTP | IC₅₀R CyP-M | IC₅₀R CyP-A |
|-----------------------------|-----------|-------------|-------------|
| CSA                         | 1.0       | 1.0         | 1.0         |
| o-MeSer-3-CS                | 0.9       | 0.4         | 0.3         |
| MeVal-4-CS                  | 1.5       | 0.8         | 0.5         |
| Val-2-MeBmt(6,7-DH)-1-CS    | 1.9       | 1.2         | 0.8         |
| Nva-2-CS                    | 2.1       | 1.1         | 1.5         |
| o-Lys(dansyl)-8-CS          | 12.4      | 5.4         | 1.8         |

**TABLE III**

Relative potency of CSA derivatives at inhibition of MTP, CyP-M, and CyP-A

The ratios were obtained by dividing the IC₅₀ of the test compound by that of CSA within each group, and were taken from Table II for CyP-M and CyP-A. For MTP, the indicated values represent the average of three independent determinations obtained by dividing the IC₅₀ of the test compound by that of CSA for the same mitochondrial preparation.
raphy protocol used by Andreeva et al. (39) for partial purification of the 22-kDa PPIase is virtually identical to that used to purify several CyP isoforms (e.g. Ref. 21 and Fig. 3). Based on these considerations, on their apparent molecular weights, and on their physico-chemical properties, we suspected that the larger proteins labeled by Andreeva et al. (39) in heart and liver subcellular fractions enriched in mitochondria are the 22- and 18-kDa endoplasmic reticulum-associated CyP-B and CyP-A, respectively.

Membrane Interactions of CyP-M and Inhibition of the MTP—The effect of acidic pH on the MTP is well documented (12). We have been able to track the inhibitory effect of protons to a matrix site (9) and to show that it is mediated by reversible protonation of histidyl residues (2). The demonstration that acidification can release CyP-M from SMP in a DPC-sensitive fashion and that the effect of H+ can be mimicked by CSA but not CSH (Fig. 2), together with our previous findings on the role of histidyl residues in MTP activity (2), acquires a particular meaning in the light of recent structural and functional studies of the interactions of immunosuppressant drugs with their intracellular receptors, the immunophilins (CyPs and FKBP5).

Yu and Fesik (40) have shown that His-126 of CyP-A, which is in close proximity to the CSA binding site, has a pKa of 6.34 in the uncomplexed protein, while in the CSA-complexed form, the pKa is shifted to 4.65. His-126 has been implied in ligand-substrate interactions and appears to contribute to the hydrophobic pocket, which is essential for substrate binding (41) and for enzyme function (42). A similar situation occurs with FKBP, the intracellular target for the immunosuppressant drugs FK506 and rapamycin, which is structurally unrelated to CyPs (43, 44) yet possesses PPIase activity and shares calcineurin activity. Indeed, MeVal-4-CS is as effective as CSA itself at pore inhibition (Ref. 25 and Table II), while the effects of FK506 on the SR-CRC can be mimicked by rapamycin, which likewise does not inhibit calcineurin (51).

The submembranal localization of CyP-M has not been studied in detail, but its copurification with matrix components (21) indicates that it is a soluble matrix enzyme. Yet, as shown here, CyP-M can associate with SMP. These observations suggest that only a fraction of CyP-M may participate in MTP modulation at any given time and that MTP opening-closure may be dependent on modulation of CyP-M binding to the membrane. Consistent with this idea, it has been shown that phenylarsine oxide, perhaps the most potent MTP agonist (1, 9, 54), increases the affinity of binding of CyP-M to mitochondrial membranes (55). Based on the findings of the present work and because of the striking analogies with SR-CRC and IP3R mentioned above (see Ref. 19 for a general discussion), we predict that the MTP-CyP-M complex will prove to be the mitochondrial homologue of the SR-CRC-FKBP12 and IP3R-FKBP12 complexes.

Inhibition of CyP-M PPIase and MTP Activities by CSA Derivatives—Independent evidence for a role of CyP-M in MTP regulation comes from the experiments with CSA derivatives, which were selected solely on the basis of their graded affinity for CyP-A. Our main findings (Tables II and III) can be summarized as follows. (i) Inhibition of MTP by CSA and its analogues correlates well with inhibition of PPIase activity of both CyP-M and CyP-A. (ii) In all cases, the best fit is observed between MTP and CyP-M. (iii) At variance of the requirements for immunosuppression, inhibition of MTP opening by CSA does not require inhibition of calcineurin since MeVal-4-CS retains the ability to inhibit the pore but not that of inhibiting calcineurin (56). (iv) Substitutions in position 8 interfere most with inhibition of MTP and of PPIase activity of CyP-M, while interactions with CyP-A are affected only minimally; this suggests that the structural differences between the two CyP isoforms go beyond the N-terminal sequences. (v) CSH does not inhibit either the MTP or PPIase activity of CyP-M.

The match between the structural requirements for inhibition of MTP and of CyP-M PPIase catalytic activity by CSA is of particular relevance when the mechanism of CyP inhibition by CSA in aqueous solution is considered. In an apparent paradox, a CSA derivative with marginal affinity for CyP-A retained a
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considerable immunosuppressive activity (57), and the x-ray crystallographic maps at 1.86 Å resolution of CyP-A complexed with CSA or with a 4-substituted CSA derivative were virtually identical despite a 4-fold higher affinity for CyP-A of the latter (58). This led to the proposal that CSA conformer equilibrates in aqueous solution rather than that differences in three-dimensional architecture govern binding to CSA (57). This hypothesis has recently been confirmed by direct measurements showing that CyP-A only recognizes a well defined conformation of CSA that exists in water prior to binding and that the rate-limiting aqueous solution rather than that differences in three-dimensional structure.

Indeed, the time required for equilibrium binding of CSA to CyP-A was as high as 1 h, and it dropped to less than 5 min (the earlier time point that could be taken) with two 3-substituted CSA analogs mimicking the conformation of CyP-A-complexed CSA (38).

Based on these findings, it appears likely that the match between inhibition of CyP-M and MTP would be even better with the purified channel, where the complexities arising from CSA partitioning between membrane and aqueous mitochondrial phases can be overcome.

Conclusions and Perspectives—We conclude that CyP-A is the main and probably the only mitochondrial receptor for CSA, that CyP-M interactions with the mitochondrial inner membrane in SMP are consistent with a key role for this immunophilin in the regulation of the MTP, and that CSA inhibition of the MTP does not require interactions with calciurein. Mitochondrial (dys)function is now increasingly considered as a key event in a variety of forms of cell death, ranging from ischemia (59–61) to excitotoxic neurodegeneration (62) to oxidant-induced stress (63) to apoptosis (64–67). Because of its exquisite sensitivity to Ca\(^{2+}\) ions, to the proton electrochemical gradient, and to oxidative stress (68), the MTP appears as a likely target on which many pathological agents or conditions may converge. The involvement of CyP-M in MTP modulation and the dissociation of MTP inhibition from the immunosuppressive effects of CSA demonstrated here offer great promise for the development of new conceptual and pharmacological tools aimed at therapeutic intervention.

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