

# Cyclophilin D Modulates Mitochondrial $F_0F_1$ -ATP Synthase by Interacting with the Lateral Stalk of the Complex\*

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Blue native gel electrophoresis purification and immunoprecipitation of  $F_0F_1$ -ATP synthase from bovine heart mitochondria revealed that cyclophilin (CyP) D associates to the complex. Treatment of intact mitochondria with the membrane-permeable bifunctional reagent dimethyl 3,3-dithiobis-propionimide (DTBP) cross-linked CyPD with the lateral stalk of ATP synthase, whereas no interactions with  $F_1$  sector subunits, the ATP synthase natural inhibitor protein IF1, and the ATP/ADP carrier were observed. The ATP synthase-CyPD interactions have functional consequences on enzyme catalysis and are modulated by phosphate (increased CyPD binding and decreased enzyme activity) and cyclosporin (Cs) A (decreased CyPD binding and increased enzyme activity). Treatment of MgATP sub-mitochondrial particles or intact mitochondria with CsA displaced CyPD from membranes and activated both hydrolysis and synthesis of ATP sustained by the enzyme. No effect of CsA was detected in CyPD-null mitochondria, which displayed a higher specific activity of the ATP synthase than wild-type mitochondria. Modulation by CyPD binding appears to be independent of IF1, whose association to ATP synthase was not affected by CsA treatment. These findings demonstrate that CyPD association to the lateral stalk of ATP synthase modulates the activity of the complex.

The mitochondrial  $F_0F_1$ -ATP synthase (complex V) is a large multisubunit complex of 600 kDa organized into a catalytic part ( $F_1$ ) and a membranous moiety ( $F_0$ ) linked by central and peripheral stalks. The enzyme is located in the inner membrane, where it catalyzes the synthesis of ATP at the expense of the proton motive force generated by the respiratory chain in the presence of oxygen (1), whereas it works in the direction

of ATP hydrolysis during anoxic conditions (2). High resolution structures of  $F_1$  sector (3, 4) and single molecule technology studies (5) established that this large complex is a rotary motor. Diffusion of protons down their electrochemical gradient drives the rotation of the c subunits ring within  $F_0$  and the rotation of the central stalk (comprising the  $F_1$   $\gamma$ ,  $\delta$ , and  $\epsilon$  subunits) inside the catalytic  $\alpha_3\beta_3$  domain of the  $F_1$  part, inducing the conformational changes in the three  $\alpha/\beta$  pairs responsible for ATP synthesis. Vice versa, ATP hydrolysis drives the central stalk rotation in the reverse direction, sustaining the formation of the proton motive force. In either case, the peripheral stalk is thought to act as a stator to counter the tendency of the  $\alpha_3\beta_3$  subcomplex to rotate with the central stalk (6).

The core mammalian enzyme consists of 15 conserved subunits, the  $F_1$  subunits  $\alpha$ – $\epsilon$  and the  $F_0$  subunits a–g, OSCP, A6L, and F6 (7). In the  $F_0$  sector, single copies of subunits b, d, F6, and OSCP form the peripheral stalk, which extends from the top of the  $\alpha_3\beta_3$  subcomplex along its external surface down into the membrane domain (6). The so-called minor subunits e, f, g, and A6L all span the membrane, but their roles as well as their exact stoichiometries are poorly defined (8). Additional subunits are species-specific. In the bovine heart enzyme, these are the coupling factor B, an activator released upon enzyme purification, which is required to maintain the energy-coupling activity of the membrane-bound ATP synthase (9), and two novel hydrophobic proteins (MLQ and AGP), which associate with the enzyme only in the presence of exogenous phospholipids and whose functions are unknown (10). Furthermore, the mitochondrial complex can associate with the inhibitor protein IF1 (11). It is well established that IF1 binding to the catalytic sector of  $F_1$  in 1:1 stoichiometry fully inhibits the ATPase activity and that such inhibition is optimal at low pH and mitochondrial membrane potential, a condition achieved in ischemia (2).

ATP synthase is commonly isolated as a functional monomer, but this appears not to be the physiological state in the membrane. Indeed, electron microscopy of native mitochondrial membranes from yeast (12), algae (13), plants (14), and mammals (15) demonstrated that ATP synthase is organized in dimers associated to form long rows of oligomers. The physiological role of these supramolecular structures is currently the subject of considerable debate. Strong evidence links supramolecular organization of ATP synthase to mitochondrial morphology because loss of cristae formation was observed under conditions that destabilize dimerization and oligomerization of

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ATP synthase (16). In addition, a recent simulation indicates that such a supramolecular assembly may favor effective ATP synthesis by causing higher local proton concentration due to local membrane curvature (15).

Mutational analyses and cross-linking experiments in yeast established that two monomers interact to form dimers through the membrane domain of the peripheral stalks and that dimers associate through interactions involving subunit e, g, and a of  $F_0$  (12, 17). In mammals, the structural properties are less characterized, although the yeast and mammalian enzymes may share similar features. In both organisms, electron microscopic studies showed that dimer/oligomer interfaces include the membranous  $F_0$  domain (15), and a variability in the shape of these supramolecular assemblies has been described (12, 18). In addition, the involvement of subunit e in oligomer formation has been demonstrated also in bovine heart mitochondria (19). An intriguing aspect of ATP synthase oligomers relates to the involvement of IF1 in their stabilization. Earlier work demonstrated that ATP synthase self-association did not require IF1 either in yeast (20) or in mammals (21), yet a recent study showed that in respiring mitochondria, IF1 overexpression stabilizes ATP synthase dimers and promotes ATP synthesis, raising questions on how IF1 functions under physiological conditions (22).

The complexity of the structural organization of the ATP synthase led us to study the involvement of chaperones in its function. We found that the peptidyl prolyl *cis-trans* isomerase cyclophilin (CyP)<sup>5</sup> D associates to the oligomeric forms of the ATP synthase and specifically interacts with OSCP, subunit d, and subunit b of the lateral stalk, decreasing its enzymatic activity, binding being favored by  $P_i$ , and that cyclosporin (Cs) A displaces CyP-D from the ATP synthase complex, resulting in stimulation of the enzyme.

## EXPERIMENTAL PROCEDURES

**Preparation of Mitochondria and MgATP Submitochondrial Particles**—Beef heart and mouse liver mitochondria were isolated in a buffer containing 250 mM sucrose, 10 mM Tris, 0.1 mM EGTA, pH 7.4, as described previously (23). MgATP submitochondrial particles (SMP) were prepared by exposure of heart mitochondria to ultrasonic energy in the presence of 15 mM  $MgCl_2$  and 1 mM ATP followed by a first centrifugation for 10 min at  $43,000 \times g$  and a second centrifugation of the supernatant for 30 min at  $100,000 \times g$  (21). Mitochondria or MgATP-SMP were suspended in buffer A (0.25 M sucrose, 10 mM Tris/MOPS, 5 mM succinate, 20  $\mu M$  EGTA, 2  $\mu M$  rotenone, pH 7.4) at a concentration of 0.8 mg/ml. When needed, 0.8–8  $\mu M$  CsA and/or 1–10 mM  $KH_2PO_4$  ( $P_i$ ) was added, and the incubation was performed at room temperature for 15 min. SMPs were then centrifuged and resuspended in proper buffers to determine ATP synthesis or hydrolysis rates as described below.

**Detergent Extraction of Mitochondrial Membranes and Gel Electrophoresis**—Pellets of mitochondria were suspended at 10 mg/ml in buffer B (1 M aminocaproic acid, 50 mM Bis-Tris, pH 7.0 as in Ref. 21), solubilized with 2% (w/v) *n*-dodecylmaltoside (DDM) or 1% (w/v) digitonin, and immediately centrifuged at  $100,000 \times g$  for 25 min at 4 °C. The supernatants were supplemented with Coomassie Blue G-250 (Serva) and rapidly applied to a one-dimensional 4–11% polyacrylamide gradient blue native gel (BNE) (24). After electrophoresis, gels were stained with Coomassie Blue, used for in-gel activity staining or prepared for SDS-PAGE as in Ref. 25. Bands corresponding to the monomeric or oligomeric form of ATP synthase were excised from one-dimensional BNE and subjected to two-dimensional resolution by 15% SDS-PAGE. Proteins in two-dimensional SDS-PAGE were (i) stained with silver or colloidal Coomassie Blue; (ii) used for Western blotting; or (iii) excised and applied to an iterative three-dimensional SDS-PAGE followed by immunoblotting. Coomassie Blue-stained gels were quantified by densitometry using ImageQuant software, version 2003.03 (Amersham Biosciences).

**Western Blotting**—Proteins separated in one-, two-, and three-dimensional SDS-PAGE were transferred electrophoretically to nitrocellulose membranes using a Mini Trans-Blot system (Bio-Rad). Western blotting was performed in phosphate-buffered saline (PBS) containing 3% nonfat dry milk with polyclonal anti- $\alpha$  and anti- $\beta$  subunit antibodies prepared in rabbits against purified bovine heart  $F_1$  (1:3,000 dilution), polyclonal anti-b subunit antibodies (a gift of Dr. John Walker, 1:10,000 dilution), polyclonal anti- $\gamma$  subunit antibodies (a gift of Dr. Daniel Br  thes, 1:10,000 dilution), monoclonal anti-CyPD antibodies from Calbiochem (1:500 dilution), monoclonal anti-OSCP, anti-d subunit and anti-IF1 inhibitor protein antibodies from Molecular Probes (1:2,000 dilution), and monoclonal anti-ANT antibodies from Santa Cruz Biotechnology (1:1,000 dilution). Immunoreactive bands were detected by enhanced chemiluminescence (Pierce). CyPD was expressed as CyPD/ $F_1$ , CyPD/IF1, and CyPD/ANT ratios of chemiluminescence intensities of the corresponding bands, and IF1 was expressed as IF1/ $F_1$  ratio (25).

**Immunoprecipitation**—As recommended by the immunocapture kit supplier, mitochondria or cross-linked samples were suspended at 5.6 mg/ml in PBS before the addition of 1% (w/v) DDM or 1% digitonin or at 4.2 mg/ml in buffer C (50 mM NaCl, 5 mM aminocaproic acid, 30 mM Tris, pH 7.4) as in Ref. 26. Samples were then incubated overnight under wheel rotation at 4 °C in the presence of anti-complex V monoclonal Ab covalently linked to protein G-Agarose beads (MS501 immunocapture kit from Mitosciences) in a ratio of 20  $\mu l$ /mg of protein. After gentle centrifugation ( $500 \times g$  for 5 min), the beads were washed twice for 5 min in a solution of 0.05% (w/v) DDM in PBS. The elution was performed in 2% (w/v) SDS for 15 min, and the collected fractions were subjected to SDS-PAGE.

**Cross-linking**—Mitochondria were suspended in PBS buffer and treated with solvent or 1–3 mM DTBP (27) for 15 min at room temperature prior to immunoprecipitation. To reduce the DTBP disulfide bond, the cross-linked immunoprecipitates were treated with 150 mM DTT for 30 min at 37 °C and separated by SDS-PAGE in the absence of reducing agents.

<sup>5</sup> The abbreviations used are: CyP, cyclophilin; Cs, cyclosporin; ANT, adenine nucleotide translocator; BNE, polyacrylamide gradient blue native gel electrophoresis; DDM, *n*-dodecylmaltoside; DTBP, dimethyl-3,3-dithiobis-propionimidate; DTT, dithiothreitol; PBS, phosphate-buffered saline; SMP, submitochondrial particles; Ab, antibody; MOPS, 4-morpholinepropanesulfonic acid; Bis-Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol.

## Cyclophilin D Interacts with Mitochondrial ATP Synthase

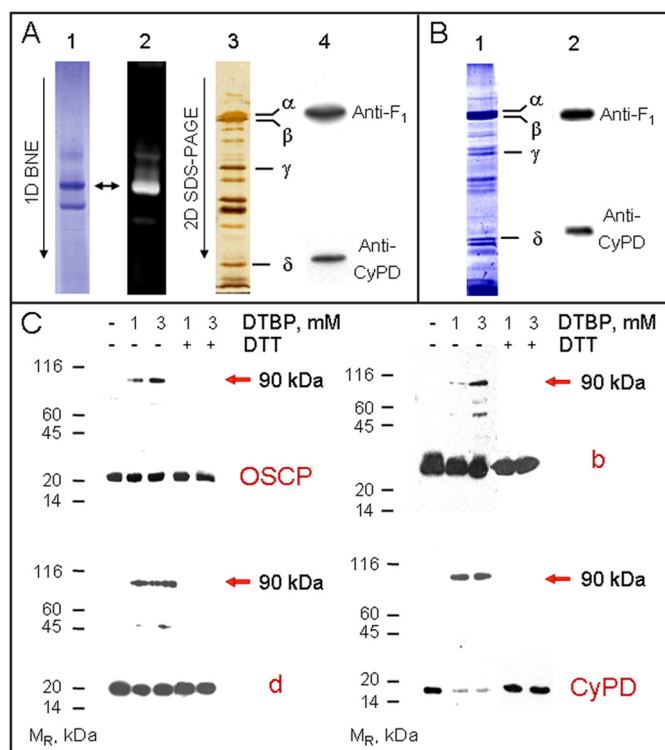
**Biochemical Assays**—Protein concentration was determined with the Lowry method for the particulate fractions (28) and with the Bradford procedure for the soluble enzymes (29). Assays of ATP synthesis and hydrolysis were performed with suspensions of MgATP-SMP (0.01  $\mu\text{g}/\mu\text{l}$ , final volume 0.2 ml and 0.1  $\mu\text{g}/\mu\text{l}$ , final volume 1 ml, respectively). Each set of samples contained the same amount of  $F_1$  as assessed by immunoblot. ATP synthesis was followed as in Ref. 30 using an ATPlite 1step luminescence ATP detection system and a luminescence reader (Victor 3-1420 multilabel counter, PerkinElmer Life Sciences). Synthesis was started by the addition of 1 mM ADP. ATP hydrolysis was followed spectrophotometrically by recording NADH oxidation at 340 nm in the presence of 2 mM ATP and of an ATP-regenerating system with or without 4  $\mu\text{M}$  oligomycin as described (31). The fraction of oligomycin-sensitive ATPase activity was always >85% and was not affected by treatment with CsA or  $P_i$ .

### RESULTS

**Endogenous CyPD Interacts with the Lateral Stalk of ATP Synthase in Bovine Heart Mitochondria**—To assess the potential interaction of CyPD with ATP synthase in the native membrane, three different approaches were used in freshly prepared bovine heart mitochondria. The first approach consisted of one-step detergent extraction of ATP synthase by DDM and separation of the native complex by one-dimensional BNE followed by two-dimensional SDS-PAGE and immunoblotting with anti-CyPD antibodies. As expected, this extraction procedure generated the monomeric form of ATP synthase on one-dimensional BNE (Fig. 1A, lane 1) because DDM yields almost lipid-free protein complexes and dissociates hydrophobic interactions (24). The monomer separated by one-dimensional BNE was identified by in-gel activity staining (Fig. 1A, lane 2), and the corresponding band was cut and subjected to two-dimensional SDS-PAGE. Silver staining clearly indicated the protein profile expected of the subunit composition of ATP synthase (Fig. 1A, lane 3). In addition, a faintly staining band located immediately above the  $\delta$  subunit was evident. Immunoblotting with anti-CyPD antibodies after two-dimensional SDS-PAGE revealed the presence of CyPD at the same position (Fig. 1A, lane 4), indicating that this protein remains associated to ATP synthase during BNE separation. The parallel detection of  $\alpha/\beta$  subunit of  $F_1$  allowed us to express CyPD content as the CyPD/ $F_1$  ratio and to follow its variations under different experimental conditions (see below).

The second approach consisted of the immunoprecipitation of ATP synthase. Mitochondria were solubilized with DDM in PBS buffer, treated with anti-complex V antibodies, and subjected to immunoblotting. Fig. 1B shows that CyPD co-immunoprecipitated with ATP synthase. The identity of CyPD was further confirmed by excising the bands from SDS-PAGE gels obtained after immunoprecipitation and from one-dimensional BNE/two-dimensional SDS-PAGE and performing iterative SDS-PAGE and immunoblotting with anti-CyPD antibodies (results not shown).

To identify the regions of ATP synthase involved in the interactions with CyPD, in the third approach, mitochondria were treated with the membrane-permeant cross-linker



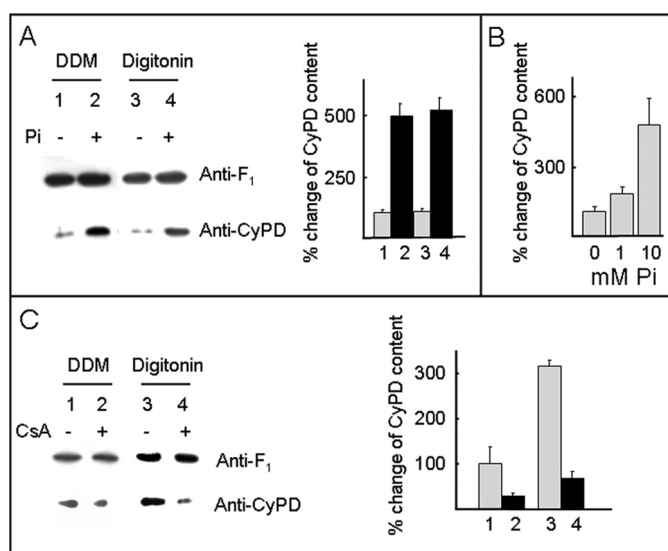
**FIGURE 1. CyPD interacts with mitochondrial ATP synthase through the OSCP and b and d subunits.** A, freshly prepared bovine heart mitochondria were solubilized in buffer B with 2% (w/v) DDM. ATP synthase (double arrow) was separated by one-dimensional BNE (1D BNE), stained by Coomassie Blue G (lane 1), and identified by in-gel ATPase assay (lane 2). The excised bands with enzymatic activity were subjected to two-dimensional SDS-PAGE (2D SDS-PAGE) and silver-stained (lane 3) or blotted on nitrocellulose for immunodetection using polyclonal Ab against  $F_1$   $\alpha/\beta$  subunits and monoclonal Ab against CyPD (lane 4). B, mitochondria were suspended in PBS buffer, treated with 1% (w/v) DDM, and immunoprecipitated with anti-complex V Ab followed by SDS-PAGE (lane 1) and immunodetection using anti- $\alpha/\beta$  or CyPD Ab (lane 2). No signal was detected when affinity-purified IgG from preimmune rabbit or mouse serum were used (results not shown). C, mitochondria suspended in PBS buffer were treated with vehicle or with the indicated concentrations of DTBP at room temperature for 15 min prior to immunoprecipitation with anti-complex V Ab. The immunoprecipitates were incubated for 30 min at 37 °C in the absence or presence of 150 mM DTT as indicated and separated by SDS-PAGE in the absence of reducing agents. Immunodetection was performed with Ab against OSCP or b or d subunits or against CyPD as indicated. Each reported blot is representative of at least two independent experiments.

DTBP before extraction and immunoprecipitation of ATP synthase with anti-complex V antibodies. DTBP is a homo-bifunctional reagent, which reacts with the primary amines of two interacting proteins at an average distance of about 8 Å (27). In addition, DTBP contains a disulfide bond that can be broken under reducing conditions, thus releasing the single components of the cross-linked products. After cross-linking and immunoprecipitation, each sample was split in two identical aliquots, which were incubated in the absence or presence of DTT. DTBP treatment generated a species of about 90 kDa that reacted with anti-OSCP, anti-b, anti-d, and anti-CyPD antibodies (Fig. 1C, arrows). A faint band with an apparent molecular mass of about 50 kDa, which corresponds to the b–d heterodimer (32), was also detected by the anti-d and anti-b antibodies (Fig. 1C). In keeping with Ref. 32, analysis of cross-linked mitochondria rather than immunoprecipitated ATP synthase by SDS-PAGE and immunoblotting revealed, in addition to the

90-kDa species, a variety of cross-linked products of b-d-OSCP subunits (data not shown). These results indicate that OSCP and b and d subunits, which are components of the lateral stalk, form a cross-linked complex with CyPD. Samples not treated with DTBP, or treated with both DTBP and DTT, displayed single bands corresponding to OSCP, b and d subunits, and CyPD (Fig. 1C). It should be noted that in the presence of DTBP, virtually all of ATP synthase-associated CyPD could be found in the cross-linked complex, whereas most of OSCP and the b and d subunits was in the monomeric form even in the absence of DTT (Fig. 1C). Finally, it should be mentioned that no interaction was observed between CyPD and  $\alpha/\beta$  or  $\gamma$  subunits of  $F_1$ , IF1, or ANT (data not shown).

**Effects of  $P_i$  and CsA on ATP Synthase-CyPD Interactions in Intact Bovine Heart Mitochondria**—The experiments carried out so far were performed in PBS. In the next set of experiments, we studied whether CyPD binding was affected by  $P_i$ , which binds to and modulates the ATP synthase (5, 33). The presence of  $P_i$  significantly increased the association of CyPD with ATP synthase, as detected in immunoprecipitates of ATP synthase with anti-complex V antibodies both in DDM and in digitonin extracts (Fig. 2A, compare lanes 1 and 3 with lanes 2 and 4, respectively; the change of CyPD content relative to  $F_1$  for each lane is given in the right part of the panel). The striking effect of  $P_i$  was also addressed in experiments where mitochondria were subjected to one-dimensional BNE/two-dimensional SDS-PAGE followed by immunoblotting, a protocol that confirmed increased binding of CyPD to the ATP synthase at increasing  $P_i$  concentrations (Fig. 2B). We next studied the effect of the CyP inhibitor CsA on the ATP synthase-CyPD complex. Freshly prepared mitochondria were treated with 0.8  $\mu\text{M}$  CsA and then extracted with DDM (which produces the monomeric form of ATP synthase) or digitonin (which allows the preparation of the well characterized oligomeric form of the enzyme (24)). The extracts were subjected to one-dimensional BNE, two-dimensional SDS-PAGE, and immunoblotting with antibodies recognizing both  $\alpha/\beta$  subunits of  $F_1$  and CyPD. A sizeable fraction of CyPD, which was otherwise associated to ATP synthase, was displaced by treatment with CsA irrespective of the detergent used (Fig. 2C). In the absence of CsA, the amount of CyPD associated with the ATP synthase was much higher after extraction with digitonin than after extraction with DDM (Fig. 2C). This is interesting because oligomers are most probably the physiological form of the enzyme (15). However, the persistent association of CyPD with the monomers, which are probably the breakdown products of oligomers, suggests a rather stable association between CyPD and the ATP synthase lateral stalk.

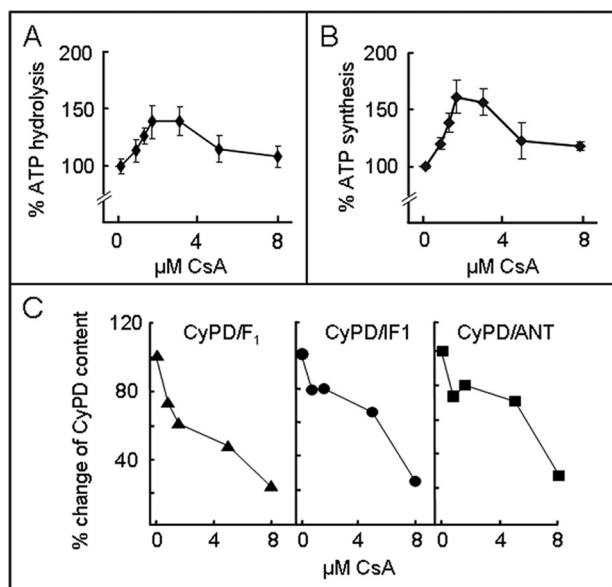
**CyPD Displacement from the Inner Membrane Activates ATP Synthase**—Considering that the lateral stalk is critical for ATP synthase catalysis, it appeared plausible that CyPD interactions could modulate enzyme activity. To test this hypothesis, we evaluated the effect of CsA in bovine heart submitochondrial particles, which contain bound CyPD that can be displaced by CsA (23). MgATP particles, which are able to sustain both ATP hydrolysis and ATP synthesis and contain IF1 (21), were suspended in succinate- and carbonylcyanide *p*-trifluoromethoxyphenyl hydrazone-containing buffer supplemented with 10 mM



**FIGURE 2.  $P_i$  and CsA modulate CyPD association to ATP synthase in intact mitochondria.** A, freshly prepared mitochondria were suspended in phosphate-free saline (lanes 1 and 3) or in PBS (lanes 2 and 4), treated with 1% (w/v) DDM (lanes 1 and 2) or 1% (w/v) digitonin (lanes 3 and 4), and immunoprecipitated with anti-complex V Ab followed by SDS-PAGE and immunoblotting with anti- $\alpha/\beta$  or CyPD Ab (representative results of one experiment out of three are shown). Each immunodetected band was analyzed by densitometry with the ImageQuant software, and the ratio between the peak area of CyPD and that of the corresponding  $\alpha/\beta$  subunits was measured and expressed relative to the ratio obtained in the absence of  $P_i$ , which was taken as 100% (graph on the right, values are mean  $\pm$  S.E. (error bars) of three independent experiments). B, mitochondria were suspended in phosphate-free buffer A (see "Experimental Procedures") alone or supplemented with 1 or 10 mM  $\text{KH}_2\text{PO}_4$ . ATP synthase oligomers were extracted with 1% (w/v) digitonin in phosphate-free buffer B, separated by one-dimensional BNE and two-dimensional SDS-PAGE, and blotted onto nitrocellulose followed by immunodetection with anti- $\alpha/\beta$  or CyPD Ab; CyPD association was quantified as in panel A (reported values are the mean  $\pm$  S.E. (error bars) of three independent experiments). C, mitochondria in buffer A supplemented with 1 mM  $\text{KH}_2\text{PO}_4$  were treated with vehicle or with 0.8  $\mu\text{M}$  CsA for 15 min at room temperature, centrifuged at  $17,000 \times g$  for 15 min at 4  $^\circ\text{C}$ , solubilized in buffer B containing 2% (w/v) DDM or 1% (w/v) digitonin, and subjected to one-dimensional BNE. DDM extracts (yielding monomers of ATP synthase) and digitonin extracts (yielding oligomers of ATP synthase) were identified by in-gel ATPase assay, excised, subjected to two-dimensional SDS-PAGE, and blotted onto nitrocellulose followed by immunodetection with anti- $\alpha/\beta$  or CyPD Ab. Lanes 1 and 2, extraction with DDM; lanes 3 and 4, extraction with digitonin (representative results of one experiment out of three are shown). The peak area of each immunodetected band was analyzed by densitometry with the ImageQuant software, and the ratio between the peak area of CyPD and that of the corresponding  $\alpha/\beta$  subunits was measured. Data were then expressed relative to the ratio obtained in lane 1, which was taken as 100% (graph on the right, where numbers refer to the lanes of the same panel; values are mean  $\pm$  S.E. (error bars) of three independent experiments).

$P_i$  to favor CyPD binding to the ATP synthase. Treatment with CsA caused a concentration-dependent increase of the rate of ATP hydrolysis, with a maximal effect at 1.6  $\mu\text{M}$ , whereas concentrations higher than 3  $\mu\text{M}$  caused a decreased rate of ATP hydrolysis (Fig. 3A). We also tested the effect of CsA on the rate of ATP synthesis in the presence of succinate and 10 mM  $P_i$  and found a very similar bell-shaped dependence on the CsA concentration with a remarkable increase of activity of about 60% (Fig. 3B). Consistent with the CyPD binding results, 10 mM  $P_i$  alone caused a 43% decrease of ATP synthesis rate (results not shown; the effect of  $P_i$  on the rate of ATP hydrolysis was not analyzed because  $P_i$  protects against the turnover-dependent deactivation of SMPs due to  $\text{ADP-Mg}^{2+}$ , resulting in enzyme stimulation (33)).

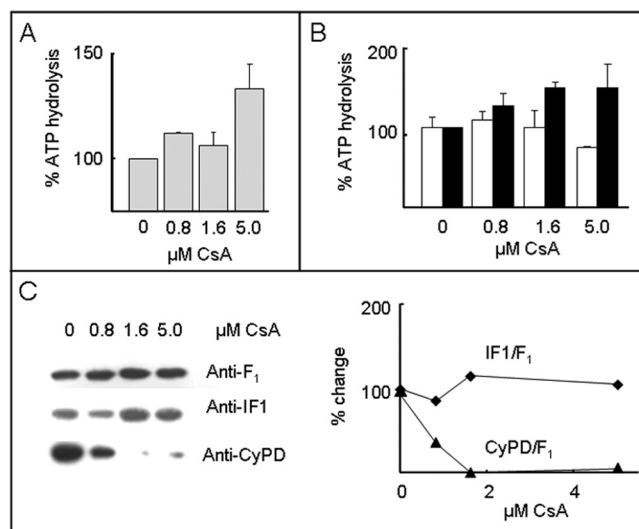
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**FIGURE 3. CsA stimulates both ATP hydrolysis and synthesis, and displaces CyPD from MgATP-SMP.** *A*, MgATP-SMP suspended in buffer A supplemented with 10 mM  $\text{KH}_2\text{PO}_4$  were treated for 15 min at room temperature with the indicated concentrations of CsA. Aliquots were then used to determine the rate of oligomycin-sensitive ATP hydrolysis at 37 °C, which was always higher than 85% of the total. The rate in the absence of CsA was taken as 100%, and reported values are mean  $\pm$  S.E. (error bars) of three independent experiments. *B*, MgATP-SMP were treated with CsA as in panel *A*, and ATP synthesis was determined at 30 °C with an ATPlite 1step luminescence detection system as described (30). The rate in the absence of CsA was taken as 100%, and reported values are mean  $\pm$  S.E. (error bars) of three independent experiments. *C*, MgATP-SMP were treated with CsA as in panel *A* and subjected to SDS-PAGE followed by immunodetection using Ab against CyPD and the  $\alpha/\beta$  subunits or  $F_1$  (left), IF1 (center), or ANT (right). Peak areas of immunodetected bands were quantified as in Fig. 2A. The ratio between the peak area of CyPD and that of each of the other proteins was determined, 100% being the ratio observed in the absence of CsA. The results are representative of three independent experiments.

Levels of CyPD associated to the inner membrane were quantified by comparing intensity of the immunoreactive band of CyPD with that of three proteins whose levels did not change upon CsA treatment, *i.e.* the  $\alpha/\beta$  subunits of  $F_1$  (Fig. 3C, left panel), the inhibitor protein IF1, which is retained in large amounts by MgATP particles (21) (Fig. 3C, middle panel), and the ANT, which is also associated to ATP synthase (34) (Fig. 3C, right panel). A bimodal decrease of the CyPD content could be observed at increasing [CsA], suggesting the existence of more than one class of binding sites for CyPD in the inner membrane (Fig. 3C, see also "Discussion").

A similar experiment was performed in isolated bovine heart mitochondria treated with the pore-forming antibiotic alamethicin (35) to maximize the rate of ATP hydrolysis. It can be seen that CsA caused a clear-cut activating effect on ATPase activity (Fig. 4A). We then prepared digitonin extracts of mitochondria in PBS and in  $\text{P}_i$ -free saline, *i.e.* two conditions under which association of CyPD with the ATPase is maximal and minimal, respectively (Fig. 2A). Remarkably, CsA increased ATPase activity in mitochondrial extracts prepared in PBS (Fig. 4B, filled bars) but not in  $\text{P}_i$ -free saline, a condition under which the activity actually decreased after treatment with 5  $\mu\text{M}$  CsA (Fig. 4B, open bars). To test whether the functional effects of CsA on ATP synthase were also matched by CyPD displacement in intact mitochondria, the digitonin extracts were sub-

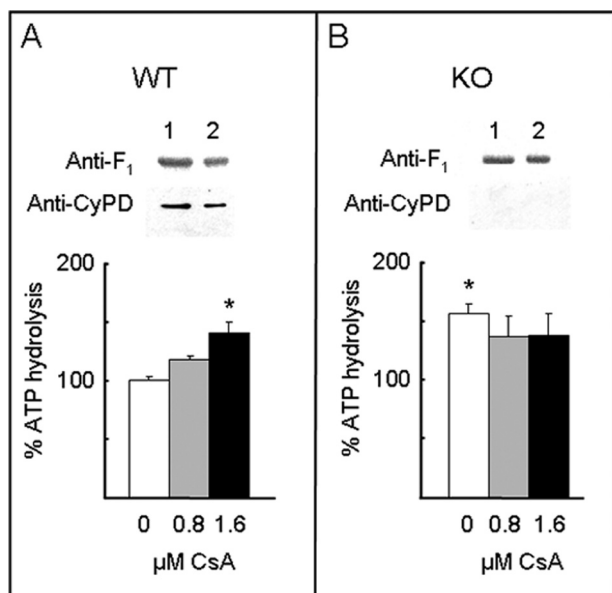


**FIGURE 4. CsA treatment increases the ATPase activity of both intact mitochondria and digitonin extracts, where it displaces CyPD.** *A*, freshly prepared mitochondria suspended in buffer A containing 10 mM  $\text{KH}_2\text{PO}_4$  were treated with the indicated concentrations of CsA, and aliquots were withdrawn to determine spectrophotometrically the maximal rate of oligomycin-sensitive ATP hydrolysis (which was always higher than 85%) in the presence of 10  $\mu\text{M}$  alamethicin. Values are mean  $\pm$  S.E. (error bars) of three independent experiments. *B*, identical aliquots of mitochondria were incubated in phosphate-free buffer C (open bars) or in PBS (closed bars) with the indicated concentrations of CsA and supplemented with 1% (w/v) digitonin followed by determination of the ATPase activity. Oligomycin sensitivity was higher than 85%, and no difference through the samples was observed. Values are mean  $\pm$  S.E. (error bars) of three independent experiments. *C*, digitonin extracts obtained using PBS buffer as in panel *B* were subjected to immunoprecipitation with anti-complex V antibodies followed by SDS-PAGE and immunodetection with antibodies against CyPD,  $F_1$   $\alpha/\beta$ , IF1, and CyP. Each immunodetected band was analyzed by densitometry with the ImageQuant software, and the ratio between the peak area of CyPD and that of the corresponding  $\alpha/\beta$  subunits in the absence of CsA was taken as 100% (graph to the right; experiments are representative of three independent determinations).

jected to immunoprecipitation with anti-complex V antibodies followed by SDS-PAGE and immunodetection with antibodies recognizing CyPD, IF1, and  $F_1$ . A CsA-dependent release of CyPD from the ATP synthase was detected, with maximum displacement at 1.6  $\mu\text{M}$  CsA (Fig. 4C). It should be noted that the concentration dependence of the effect of CsA in intact mitochondria and immunoprecipitates after digitonin extracts cannot be readily compared.

No significant variation in IF1 content was instead observed at any CsA concentration (Fig. 4C). This finding indicates that IF1 is not displaced by CsA and is therefore not responsible for the increased ATPase activity. Taken together, these results support the conclusion that CyPD release from the lateral stalk of ATP synthase activates the enzyme.

**Lack of Activation of ATP Hydrolysis by CsA in CyPD-null Liver Mitochondria**—The availability of *Ppif*<sup>-/-</sup> mice selectively lacking CyPD (36) allowed us to further test the hypothesis that CyPD regulates ATP synthase. Wild-type mouse liver mitochondria incubated in the presence of alamethicin displayed an increased rate of ATP hydrolysis of almost 50% at 1.6  $\mu\text{M}$  CsA (Fig. 5A). Remarkably, the ATPase activity of CyPD-null mitochondria was 50% higher than that of wild-type mitochondria, and CsA had no effect on the rate of ATP hydrolysis (Fig. 5B).



**FIGURE 5. Effect of CsA on ATPase activity in wild-type and CyPD-null mouse liver mitochondria.** Freshly prepared liver mitochondria from wild-type (WT, A) and CyPD-null mitochondria (KO, B) were suspended in buffer A containing 10 mM  $\text{KH}_2\text{PO}_4$  and treated with the indicated concentrations of CsA. Aliquots were withdrawn to determine (i) the amount of ATPase present by immunoblotting with anti-F<sub>1</sub>  $\alpha/\beta$  Ab (30  $\mu\text{g}$  of protein in lanes 1 and 15  $\mu\text{g}$  of protein in lanes 2, upper part of both panels) and (ii) ATP hydrolysis rate in the presence of 10  $\mu\text{M}$  alamethicin (lower part of both panels). Oligomycin sensitivity was higher than 85%, and no difference through the samples was observed. Reported values are mean  $\pm$  S.E. (error bars) of three independent experiments. In both panels, asterisks denote  $p < 0.05$  relative to the 100% value measured in wild-type mitochondria.

## DISCUSSION

This study has documented that CyPD associates to the mitochondrial ATP synthase in intact bovine heart mitochondria and that this interaction is favored by  $\text{P}_i$  and counteracted by CsA. The association of CyPD with the ATP synthase has been demonstrated by BNE and immunoprecipitation after extraction with both DDM and digitonin and by cross-linking experiments with DTBP. Remarkably, treatment with the CyPD inhibitor CsA displaced CyPD and increased activity in the direction of both ATP synthesis and hydrolysis. Finally, liver mitochondria isolated from *Ppif*<sup>-/-</sup> mice were not affected by CsA and displayed a higher rate of ATP hydrolysis than mitochondria from wild-type mice. Taken together, these findings represent a strong indication that the stimulation of ATPase activity by CsA is indeed caused by the displacement of CyPD.

Cross-linking with DTBP indicates that the contact area between CyPD and ATP synthase involves the lateral stalk. Indeed, the ~90 kDa cross-linked complex containing CyPD also contained the subunits OSCP, b and d; and it is interesting that subunit b of the ATP synthase was recently detected among CyPD binding proteins in affinity purification-based protocols.<sup>6</sup> We note that the apparent mass of the cross-linked complex matches quite well the sum of the molecular masses of CyPD, OSCP, and b and d subunits in a 1:1:1:1 ratio.

Our experiments with DTBP revealed that a significant fraction of OSCP and b and d subunits did not react with CyPD,

whereas basically all ATP synthase-associated CyPD molecules were involved in the formation of the cross-linked species (Fig. 1C). This observation suggests that there may be fewer CyPD molecules than ATP synthase units and/or that not all ATPase complexes bind CyPD under our experimental conditions. Thus, the functional effect of CyPD binding to individual complexes may be more marked than the average measured in whole mitochondria.

Modulation by CyPD appears to be independent of IF1 release as the content of the latter did not change upon CsA treatment. It should also be noted that CyPD binding affects both the forward and the reverse catalysis. This is at variance from IF1, which binds to one  $\beta$  subunit and inhibits ATP hydrolysis (11). On the other hand, we note that CsA increased ATP synthesis more than ATP hydrolysis, a finding that may actually be related to IF1. Indeed, IF1 binding could partially mask the effect of CsA during ATP hydrolysis, an effect that would be lost during synthesis when IF1 is expelled from the ATP synthase complex (11).

In SMPs, stimulation of both ATP hydrolysis and synthesis rates peaked between 1.6 and 3  $\mu\text{M}$  CsA (Fig. 3, A and B), whereas a further increase of concentration rather caused a decreased enzyme activity. The inhibitory effect of CsA on respiration (results not shown) can explain the decreased rate of synthesis but not of hydrolysis. We therefore suspect that a toxic effect on the ATP synthase may take place at higher concentrations of CsA, possibly due to the overproduction of reactive oxygen species (37, 38), which are known to inactivate the enzyme (39). It should be noted that displacement of CyPD from SMPs increased further at 5 and 8  $\mu\text{M}$  CsA (Fig. 3C), suggesting the existence of more than one class of binding sites for CyPD. Inspection of the graphs suggests that the stimulatory effect on the ATP synthase correlates with displacement from sites of higher affinity.

Involvement of the lateral stalk in the interactions of CyPD with the ATP synthase may also bear on a different aspect of mitochondrial physiology. The lateral stalk (the stator) is thought to prevent the futile rotation of the  $\alpha_3\beta_3$  hexamer with the central stalk (6) but also to mediate the formation of ATP synthase dimers and oligomers (12). In native membranes, these supramolecular forms optimize the enzyme catalytic performance (15, 22) and display a wide variability in the angle formed by the monomers (12). It is tempting to speculate that CyPD displacement by CsA activates the enzyme by favoring the formation of oligomers characterized by a higher activity. Conversely, CyPD binding might switch the enzyme to a different oligomeric assembly (*i.e.* a state with a different angle between monomers) with lower catalytic activity.

Millimolar [ $\text{P}_i$ ] favored binding of CyPD. This event may be related to the binding of  $\text{P}_i$  to ATP synthase at the catalytic site (5), which mediates various conformational effects and occurs in the millimolar range when the enzyme is not under continuous turnover (33). This is the case under our experimental conditions where  $\text{P}_i$  was added in the absence of adenine nucleotides. Because OSCP undergoes considerable stress upon binding/release of nucleotides during the catalytic cycle (40), we speculate that  $\text{P}_i$  binding might also induce conformational

<sup>6</sup> C. Baines, personal communication.

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changes in OSCP that could be transferred to its binding partners, including CyPD.

In the absence of  $P_i$ , the binding of CyPD is negligible. We therefore suspect that inhibition of the  $F_0F_1$  activity by CyPD only occurs when the enzyme hydrolyzes ATP and  $[P_i]$  increases, like in hypoxia and ischemia-reperfusion injury. Thus, inhibition by CyPD may synergize with IF1 and reduce energy dissipation.

It should be mentioned that CyPD,  $P_i$ , and CsA also modulate the permeability transition pore, an inner membrane channel of unknown composition that plays a major role in disease (41, 42). A recent finding indicates that CyPD ablation (or treatment with CsA) unmasks a binding site for  $P_i$ , which is the actual pore inhibitor in the presence of CsA (43). Whether the interactions of CyPD with the ATP synthase described here are related to the permeability transition is an intriguing possibility that will require further study.

CyPD is a member of the CyP family of peptidyl prolyl *cis-trans* isomerases (44), which includes 17 unique proteins (45). CyPs are chaperones, but we currently have no evidence that this activity may be related to assembly of the ATP synthase, which is not affected in CyPD-null mitochondria (results not shown). Enzymatic activity is inhibited by binding of the inhibitory ligand CsA (46). It remains to be established whether the inhibitory effect on the ATP synthase described here is related to the catalytic activity of CyPD, an issue that will require the definition of the interacting residues on CyPD and the ATP synthase, and the use of catalytically inactive CyPD variants.

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