We have investigated the role of arginine residues in the regulation of the mitochondrial permeability transition pore, a cyclosporin A-sensitive inner membrane channel. Isolated rat liver mitochondria were treated with the arginine-specific chemical reagent 2,3-butanedione or phenylglyoxal, followed by removal of excess free reagent. After this treatment, mitochondria accumulated Ca\(^{2+}\) normally, but did not undergo a permeability transition following depolarization, a condition that normally triggers opening of the permeability transition pore. Inhibition by 2,3-butanedione and phenylglyoxal correlated with matrix pH, suggesting that the relevant arginine(s) are exposed to the matrix aqueous phase. Inhibition by 2,3-butanedione was potentiated by borate and was reversed upon its removal, whereas inhibition by phenylglyoxal was irreversible. Treatment with 2,3-butanedione or phenylglyoxal after induction of the permeability transition by Ca\(^{2+}\) overload resulted in pore closure despite the presence of 0.5 mM Ca\(^{2+}\). At concentrations that were fully effective at inhibiting the permeability transition, these arginine reagents (i) had no effect on the isomerase activity of cyclophilin D and (ii) did not affect the rate of ATP translocation and hydrolysis, as measured by the production of a membrane potential upon ATP addition in the presence of rotenone.

We conclude that reaction with 2,3-butanedione and phenylglyoxal results in a stable chemical modification of critical arginine residue(s) located on the matrix side of the inner membrane, which, in turn, strongly favors a closed state of the pore.

Mammalian mitochondria contain an inner membrane channel, the permeability transition pore (PTP)\(^1\), that, when fully open, permits free diffusion of solutes with a molecular mass of up to ~1500 Da (1, 2). The PTP is controlled by several ligands as well as by the ΔΨ across the inner membrane; high values of ΔΨ favor the closed state, whereas a decrease in ΔΨ may increase the open probability (3). High matrix Ca\(^{2+}\) concentrations, P\(_r\), and oxidation of intramitochondrial pyridine nucleotides promote pore opening, whereas ADP, H\(^{+}\), and CsA cause inhibition (1, 2). CsA binds to mitochondrial matrix CyP-D (4), which may cause pore inhibition by releasing CyP-D from its putative binding sites on the pore (5, 6). Based on the effect of atracyloside and bongkrekate on the permeability transition (7) and based on the finding that the ANT may form a Ca\(^{2+}\)-dependent large conductance channel in phospholipid bilayers (8), it has been suggested that the pore may be formed by the ANT (7).

The physiological function of the permeability transition is not known, but circumstantial evidence suggests that it is involved in Ca\(^{2+}\) homeostasis (9, 10) and linked to cell death. This notion has gained momentum with the findings that (i) Bax and Bcl-\(\text{XL}\), two channel-forming proteins of the Bel-2 superfamily localized mainly to mitochondria (11, 12), may affect the PTP and therefore release of the apoptosis-inducing factor, a mitochondrial caspase (13) (see Ref. 14 for a review); and that (ii) mitochondrial release of cytochrome c (15, 16), an early event in apoptosis, may involve the permeability transition (see Ref. 17 for a review). Presently, however, further investigations into the function of the pore and its relation with apoptosis are hampered by lack of structural information on the PTP and by lack of pore-specific inhibitors. Indeed, not even CsA is selective for the PTP at the cellular level (see discussion in Refs. 1 and 9), and its inhibitory effect in isolated mitochondria is transient (18).

The use of selective chemical reagents in intact mitochondria has proven of great value in characterizing functionally important amino acid residues. With this approach, we have identified (i) a dithiol that controls the pore conformation through disulfide interconversions (19, 20) and (ii) a matrix histidine that appears to control CyP-D binding (6, 21). Prompted by the finding that PGO inhibits uncoupler-induced PTP opening (22), we carried out a thorough investigation on the effects of arginine-selective reagents on the permeability transition. We show that treatment of isolated rat liver mitochondria with BAD or PGO, followed by removal of excess free reagent, inhibits the permeability transition following depolarization. Remarkably, modification with PGO or BAD was sufficient to induce pore closure even at high (0.5 mM) free Ca\(^{2+}\) concentrations, implying a mechanism of inhibition different from that of both ADP and CsA (23). Treatment with PGO or BAD at concentrations providing effective inhibition of the PTP (i) had no major effect on mitochondrial energy conservation, (ii) did...
not affect the enzymatic activity of CyP-D, and (iii) did not modify the rate of ATP translocation and hydrolysis. We conclude that treatment with PGO and BAD results in a stable chemical modification of critical arginine residue(s), which, in turn, strongly favors a closed state of the pore.

MATERIALS AND METHODS

Rat liver mitochondria were prepared from male albino Wistar rats as described previously (24). Preincubation of mitochondria with PGO or BAD was carried out at room temperature for 15 min in 250 mM sucrose, 20 mM EGTA/Tris, 10 mM Hepes-KOH (pH 8.0). In some experiments, the medium was supplemented with 10 mM borate. (Preincubation with borate in the absence of arginine reagents had no effect on the permeability transition (data not shown).) The reaction was terminated by adjusting the pH to 6.8 with Hepes and by cooling to 4 °C, whereasupon mitochondria were sedimented by centrifugation at 8000 g for 10 min. The pellet was resuspended at 1 mg of protein/ml in 250 mM sucrose, 20 mM EGTA/Tris, 10 mM Hepes-KOH (pH 8.0), 0.5 mM CaCl₂, and 10 mM Tris-HCl (pH 7.4). The centrifugation was repeated once, and the mitochondria were finally resuspended at ~50 mg of protein/ml in 250 mM sucrose, 0.1 mM EGTA, and 10 mM Tris-HCl (pH 7.4).

Unless otherwise stated, the standard assay medium contained 250 mM sucrose, 10 mM Hepes/Tris (pH 7.4), 5 mM succinate, 1 mM P/Tris, and 2 μM rotenone. The slight inhibitory effect of PGO on uncoupled respiration has been described previously (22), and BAD was found to have similar effects, although at somewhat higher concentrations (data not shown). Mitochondrial swelling, Ca²⁺ transport, ΔΨ, oxygen consumption, and matrix pH were measured as described previously (22, 25). Peptidylprolyl cis,trans-isomerase activity was measured spectrophoto metrically as described (26), except that the assay medium was supplemented with 0.1% Nonidet P-40. ATP hydrolysis was measured based on the oxidation of NADH at 370 nm with a coupled enzyme assay in 250 mM sucrose, 10 mM Hepes/Tris (pH 7.0), 2 mM MgCl₂, 1 mM ATP, 1 mM phosphoenolpyruvate, 1 mM NADH, 0.1 mM EGTA, 0.5 unit/ml pyruvate kinase, and 0.5 unit/ml lactate dehydrogenase.

Chemical modification of mitochondria that had undergone permeability transition (see Fig. 3) was performed as follows. Mitochondria were suspended at 1 mg of protein/ml in 30 mM sucrose, 10 mM Hepes-KOH (pH 7.8), 0.5 mM CaCl₂, and 10 mM arsenazo III, and the permeability transition was monitored spectrophotometrically. When the whole population had undergone permeability transition (i.e. when the absorbance had stabilized at ~50% of its initial value, which took ~30 min), arginine reagents were added. After 15 min of incubation, mitochondria were loaded onto a Sephadex G-100 column equilibrated with 30 mM sucrose, 10 mM Hepes/Tris (pH 7.8), and 0.5 mM CaCl₂ recovered in the flow-through fraction; and used immediately as shown in the experiments of Fig. 3 at a concentration of 0.3 mg/ml. Chemical modification of recombinant CyP-A with PGO was carried out in 250 mM sucrose, 20 mM EGTA, and 10 mM Hepes-KOH (pH 8.0). Following modification, isomerase activity was measured at a protein concentration of 20 μg/ml.

For electron microscopy, mitochondria suspended in the standard assay medium were fixed by mixing with an equal volume of 2.5% glutaraldehyde, followed by centrifugation at 10,000 × g for 3 min. Samples were post-fixed with osmium tetroxide, dehydrated, and embedded in epoxy resin. Thin sections were stained with lead citrate and uranyl acetate. CsA and human recombinant CyP-A were a kind gift of Dr. Boland Wenger (Novartis, Basel, Switzerland). All other chemicals were purchased from Sigma.

RESULTS

To explore the role of arginine residues in the PTP, mitochondria were allowed to react with 2 mM BAD or PGO for 15 min, followed by removal of excess reagent by centrifugation. Both control and BAD- or PGO-treated mitochondria readily accumulated Ca²⁺ from the medium following energization (Fig. 1). After completion of Ca²⁺ uptake, ruthenium red was added to prevent efflux of Ca²⁺ by reversal of the unipporter, and the permeability transition was induced by addition of the protonophore FCCP. Control mitochondria rapidly released Ca²⁺ (Fig. 1, trace a) and underwent swelling due to sucrose diffusion (electron micrographs in row a). In the presence of CsA, both Ca²⁺ release and swelling were inhibited (trace b and row b), indicating that the PTP is the main pathway for Ca²⁺

Fig. 1. Inhibition of the PTP by BAD and PGO. Control mitochondria (traces a and b) and mitochondria pretreated with 2 mM PGO (trace c) or BAD (trace d) were suspended at 0.3 mg/ml in 250 mM sucrose, 10 mM Hepes/Tris (pH 7.4), 1 mM P/ 20 μM Ca²⁺, 15 μM arsenazo III, and 2 μM rotenone. Light absorption was measured at the wavelength couple 665/685 nm. Where indicated, 5 mM succinate (succ), 0.1 μM ruthenium red followed by 1.0 μM FCCP (RR + FCCP), and 1 μM A23187 (A₂₃) were added. In trace d, 1 μM CsA was present. Electron micrographs (rows a–d) show the mitochondrial ultrastructure prior to addition of succinate (0 min) and after addition of ruthenium red and FCCP (15 min) under the conditions of traces a–d, respectively.
and sucrose fluxes under these conditions. In both PGO- and BAD-treated mitochondria, Ca\(^{2+}\) release was almost completely inhibited (traces c and d, respectively), and mitochondria remained impermeable to sucrose (rows c and d, respectively), indicating that they did not undergo a permeability transition. Neither BAD nor PGO inhibited the permeability transition when added directly to the cuvette just before mitochondria (data not shown). These data suggest that inhibition of the permeability transition was due to covalent protein modification.

To approach the problem of specificity, we investigated if the modification had the characteristic features of the arginine-carbonyl addition reaction. Fig. 2 shows that the inhibitory effect of BAD on the rate of Ca\(^{2+}\) efflux was potentiated by borate. In the presence of 10 mM borate, half-maximal inhibition of the permeability transition was observed at 0.2 mM BAD (closed circles), whereas in the absence of borate, it increased to 1 mM BAD (open circles), consistent with the stabilizing effect of borate ions on the reaction product of BAD and arginine residues in proteins (27). To assess whether inhibition was reversible, we incubated BAD- and PGO-treated mitochondria at room temperature for up to 3 h after removing excess reagent and before inducing the permeability transition (Fig. 2). In PGO-treated mitochondria, the rate of Ca\(^{2+}\) release was essentially stable with time (inset, open triangles), whereas BAD-treated mitochondria released Ca\(^{2+}\) faster at increasing incubation times (inset, closed circles), indicating a recovery of the activity of the permeability transition distinguishable from the smaller increase observed in untreated mitochondria (inset, closed squares).

To rule out an effect on other protein(s) that indirectly exert control over the permeability transition by modulating mitochondrial ion or metabolite concentrations or \(\Delta\Psi\), we studied the effects of PGO and BAD in mitochondria that had already undergone permeability transition, i.e. after they had lost their content of ions and small molecules. Under these conditions, any effect of arginine reagents on the permeability transition is likely to reflect a change in the conformation of the pore (28). Respiring mitochondria suspended in a hypotonic sucrose-based medium were incubated in the presence of 0.5 mM Ca\(^{2+}\) to induce the permeability transition, and then aliquots were either (i) treated with PGO or BAD, followed by removal of excess reagent by gel filtration, or (ii) untreated and subjected to the same gel filtration protocol (see “Materials and Methods” for details). Fig. 3 shows that addition of 4% polyethylene glycol 8000 to untreated mitochondria resulted in a rapid increase in light scattering, due to shrinkage of mitochondria following sucrose diffusion through the open pore(s) (trace a) (see also Ref. 29). When Ca\(^{2+}\) was chelated by 2 mM EGTA prior to the addition of polyethylene glycol, only a small change in light scattering followed the addition of polyethylene glycol 8000 to control mitochondria, showing that removal of Ca\(^{2+}\) caused pore closure (trace b). Remarkably, PGO- and BAD-treated mitochondria underwent only a small shrinkage upon addition of polyethylene glycol, suggesting that the PTP had been locked in the closed conformation despite the presence of 0.5 mM Ca\(^{2+}\) (trace c). It should be noted that both PGO and BAD inhibited the pore to a comparable degree compared with the combination of CsA and ADP (trace d), whereas neither CsA nor ADP alone was able to inhibit the pore under these conditions (data not shown). These results indicate that the PGO- and BAD-reactive arginine(s) are directly involved in the PTP open-closed transitions, which under these conditions are independent of \(\Delta\Psi\) or of any regulatory compounds like ADP, Mg\(^{2+}\), or pyridine nucleotides. To our knowledge, the arginine modification described here is the only condition identified so far that allows pore closure at high Ca\(^{2+}\) concentrations in the absence of ADP.

We next addressed the question of whether the relevant arginine(s) are localized on the external side or on the matrix side of the inner membrane. At millimolar concentrations, both...
PGO and BAD partition about equally in water/hexane (data not shown), suggesting that their matrix and medium concentrations were the same. Furthermore, passive swelling experiments showed that borate equilibrated rapidly (within seconds) across the inner membrane (data not shown). Thus, the distribution of the reagents provided no clue regarding the membrane topology of the reactive arginine(s). Therefore, we studied whether the modification was pH-dependent and whether it correlated with the pH of the mitochondrial matrix or of the incubation medium. To modulate matrix and medium pH independently, we incubated de-energized mitochondria in isotonic sucrose or KCl medium and performed chemical arginine modification at external pH values ranging between 7.4 and 8.4.² Fig. 4 shows that modification with BAD (or PGO (data not shown and Ref. 22)) was more efficient at alkaline pH. However, the PTP was more effectively inhibited when mitochondria were modified in KCl than in sucrose medium at all values of external pH (Fig. 4A). Nevertheless, when the data were replotted as a function of matrix pH measured with 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein, a direct correlation was obtained between the pH and the degree of PTP inhibition (Fig. 4B). These results demonstrate that chemical modification with BAD and PGO is pH-dependent, support the possibility that protonation of the guanidino group is at equilibrium matrix pH, and thus suggest that the relevant arginines face the matrix.

CsA is thought to inhibit the permeability transition by binding to the active site of matrix CyP-D, a peptidylprolyl cis,trans-isomerase, thereby releasing it from the putative binding sites on the pore (5, 6). Fig. 5 shows that the total peptidylprolyl cis,trans-isomerase activity of mitochondria (largely due to CyP-D) was unaffected by treatment with 2 mM BAD or PGO (i.e., a concentration causing >90% inhibition of the permeability transition) and that CyP-A was only 10% inhibited at 10 mM PGO (inset). On the other hand, the peptidylprolyl cis,trans-isomerase activity was fully inhibited by CsA in control mitochondria as well as in BAD- or PGO-treated mitochondria. These data demonstrate that the mechanism by which BAD and PGO inhibit the permeability transition is not related to inhibition of CyP-D.

Several lines of evidence suggest that the PTP and the ANT may be functionally linked or even structurally identical (7, 8, 31). We therefore investigated whether BAD or PGO treatment inhibits the ANT. ATP/ADP exchange across the inner membrane was measured as the rate of ATP hydrolysis by intact de-energized mitochondria. Fig. 6 shows that the rate of atracyloside-sensitive ATP hydrolysis was essentially unaffected by concentrations of BAD and PGO relevant for pore inhibition. Furthermore, treatment with 1 mM PGO or BAD (i.e., a concentration that caused >90% inhibition of the PTP) only marginally affected the development of the ΔΨ produced by ATP hydrolysis in the presence of rotenone (Fig. 7). Thus, full inhibition of the PTP by arginine reagents occurred under conditions where the activity of the ANT and of the F₁F₀-ATPase remained largely unaffected.

**DISCUSSION**

**General Considerations**—We have characterized the effects of two specific arginine reagents, BAD and PGO, on the mitochondrial PTP. Modification with both reagents (i) prevents the permeability transition, which is otherwise triggered by membrane depolarization after Ca²⁺ accumulation (Fig. 1), and (ii) allows PTP closure after permeability transition induction by Ca²⁺ overload, despite the presence of 0.5 mM Ca²⁺ and the depletion of pyridine and adenine nucleotides. BAD or PGO treatment alone is as effective at inducing pore closure as 1 μM CsA and 0.5 mM ADP together, but is more potent than CsA or ADP alone (Fig. 3). Inhibition by BAD and PGO can be observed at concentrations that do not affect the activity of

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² Mitochondria lose a fraction of matrix K⁺ during isolation in a low K⁺ medium due to the activity of the constitutive K⁺/H⁺ antiporter (30). As a result, matrix pH of nonenergized mitochondria in sucrose medium is ~0.7–1.0 unit lower than external pH (e.g., Ref. 21), whereas in KCl medium, matrix and external pH coincide, as revealed by direct measurements with 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (data not shown).
BAD and PGO React with Arginine Residues—The guanidino group of arginine can be specifically modified by the dialdehydes BAD and PGO to form stable derivatives (32, 33). It was necessary, however, to characterize the reactions of both compounds with mitochondria to exclude unspacious effects. Our results indicate that the reaction followed the predicted pattern of the arginine-dicarbonyl addition. (i) Modification with BAD was stimulated by borate and could be reversed upon its removal, whereas modification with PGO was borate-independent and essentially irreversible (Fig. 2). These findings are consistent with the known effect of borate ions, which stabilize the reaction product of BAD and arginine residues in proteins (27). (ii) The reaction between PGO and arginine results in the formation of a stable adduct, whereas the complex between BAD and the guanidino group slowly dissociates in the absence of borate, resulting in the recovery of intact arginines (34). According to this paradigm, we observed that PGO inhibition was essentially irreversible, whereas PTP opening could be recovered after prolonged incubation of BAD-treated mitochondria after washing (Fig. 2). It remains unclear why PTP opening was easier after prolonged incubations even with control mitochondria, but this event is probably related to loss of pore inhibitory factors like Mg\(^{2+}\) and adenine nucleotides. (iii) Chemical modification of arginine is pH-dependent since only the deprotonated form of the guanidino group reacts with dialdehydes (35); accordingly, we found that the reaction of both PGO and BAD was favored at alkaline pH values. The extent of modification (as measured from pore inhibition) correlated better with matrix pH than with external pH, suggesting that the relevant arginine(s) are located in the matrix.

Interesting questions arise as to the physical properties of the reactive arginine residue(s) involved in pore modulation. The reactivity of dialdehydes toward the guanidino group is primarily determined by the arginine’s pK\(_a\) (which is ~12 in free arginine), the protonated form being inert (35). We therefore predict that the arginine residue(s) involved in reaction with BAD and PGO must have a much lower pK\(_a\), possibly because of the influence of a highly positively charged environment in the protein. Furthermore, the site must be readily accessible to the (matrix) aqueous phase and sufficiently exposed to the protein surface to accommodate the two bulky phenyl groups of the PGO-arginine adduct (32).

Selectivity of the Effects of PGO and BAD on the Permeability Transition—The molecular nature of the PTP remains unsolved. The protein linked to the PTP by the strongest (yet indirect) evidence is CyP-D (4, 36), which is suspected to mediate the inhibitory effects of CsA (5, 6). The active site of cyclophilins has one highly conserved arginine residue (Arg-54 and Arg-96 in rat CyP-A and CyP-D, respectively) located in a positively charged environment (37). This represents a logical target for PGO and BAD, yet we found that both rat CyP-D and human CyP-A remained active after treatment with high concentrations of PGO and BAD (Fig. 5), proving that the arginine(s) involved in pore modulation are not located in the active site of CyP-D. This interpretation is consistent with the experiments of Fig. 3, which suggest that the mechanism of PTP inhibition by BAD and PGO differs from that of CsA.

The ANT is often called in cause as a putative PTP component. The ANT affects the permeability transition in a strictly conformation-specific manner. The atracylylate-bound form (c conformation) favors the permeability transition, whereas the bongkrekate-bound form (m conformation) favors pore closure. In either case, the ANT is inhibited and locked into one of its conformational states, which might affect the pore indirectly through an effect on the surface potential (1, 18). We found that concentrations of BAD and PGO causing effective pore inhibition did not cause changes in the atracylyside-sensitive ATP/ADP exchange (Fig. 6). We conclude that the critical arginines are not located on the translocase.
Finally, treatment with PGO and BAD affected energy conservation only marginally since only uncoupled respiration was inhibited (22), explaining why the rate of Ca\(^{2+}\) uptake, but not its extent, is affected by BAD and PGO (Fig. 1). We also note that membrane permeability was unaffected by these reagents since neither basal respiration (22) nor development and maintenance of ∆Ψ were affected in BAD- or PGO-treated mitochondria (Fig. 7).

We conclude that the effects of BAD and PGO can be traced to a rather selective effect on the PTP. We are currently devising protocols for selective labeling of mitochondrial membrane proteins with [\(^{14}\)C]PGO and for using these novel biochemical tools to inhibit PTP opening in intact cells.

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