Three Classes of Ubiquinone Analogs Regulate the Mitochondrial Permeability Transition Pore through a Common Site*

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To identify the structural features required for regulation of the mitochondrial permeability transition pore (PTP) by ubiquinone analogs (Fontaine, E., Ichas, F., and Bernardi, P. (1998) J. Biol. Chem. 40, 25734–25740), we have carried out an analysis with quinone structural variants. We show that three functional classes can be defined: (i) PTP inhibitors (ubiquinone 0, decylubiquinone, ubiquinone 10, 2,3-dimethyl-6-decyl-1,4-benzoquinone, and 2,3,5-trimethyl-6-geranyl-1,4-benzoquinone); (ii) PTP inducers (2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone and 2,5-dihydroxy-6-undecyl-1,4-benzoquinone); and (iii) PTP-inactive quinones that counteract the effects of both inhibitors and inducers (ubiquinone 5 and 2,3,5-trimethyl-6-(3-hydroxyisoamyl)-1,4-benzoquinone). The structure-function correlation indicates that minor modifications in the isoprenoid side chain can turn an inhibitor into an activator, and that the methoxy groups are not essential for the effects of quinones on the PTP. Since the ubiquinone analogs used in this study have a similar midpoint potential and decrease mitochondrial production of reactive oxygen species to the same extent, these results support the hypothesis that quinones modulate the PTP through a common binding site rather than through oxidation-reduction reactions. Occupancy of this site can modulate the PTP open-closed transitions, possibly through secondary changes of the PTP Ca²⁺ binding affinity.

Regulation of ion fluxes across the inner mitochondrial membrane is essential both for metabolic regulation and for energy conservation. The inner membrane possesses an intrinsically low permeability to ions and solutes, which allows energy conservation in the form of a proton electrochemical potential difference (1), and a set of channels and transporters that regulate ion fluxes and volume homeostasis (see Ref. 2 for a recent review). However, mitochondria in vitro can easily undergo a permeability increase to solutes with molecular masses of about 1,500 Da or lower, which is followed by deenergization, disruption of ionic homeostasis, and swelling, the so-called permeability transition (PT).1 The PT has long been studied as a target for mitochondrial dysfunction in vivo, particularly in the context of ischemia-reperfusion injury (3–5). Interest in the PT as a mediator of cell death has more recently been extended to programmed cell death, a process in which mitochondria are involved through release of cytochrome c (6) and of other proteins like apoptosis-inducing factor (7), procaspases 3 and 9 (8, 9), and through regulation of the levels of cellular ATP (10). It is widely assumed that the PT is due to opening of a proteinaceous pore, the PTP, whose molecular identity remains debated (see Refs. 2 and 11 for discussion). Recent results suggest that the PTP may open under physiological conditions (12), supporting the hypothesis that it may serve as a mitochondrial Ca²⁺ release channel (13).

Ca²⁺ is the single most important factor for PTP opening in vitro, but the PTP open-closed transitions are also modulated by a large variety of physiological factors and drugs. Among these, the PTP inhibitor CsA has become the standard diagnostic tool for the characterization of the PTP in isolated mitochondria, in living cells and organs, and in vivo (see Ref. 2 for review). However, CsA does not selectively act on mitochondria, and it inhibits other cellular functions that depend on Ca²⁺ and/or on calcineurin (14). Furthermore, PTP inhibition by CsA is transient (15) and can be overcome by increasing Ca²⁺ loads (16). Even in the most successful cases of in vivo protection by CsA in brain models of disease, exclusion of the drug by the blood-brain barrier may limit its usefulness (17–19).

We have long been involved in the study and characterization of PTP inducers and inhibitors, with the long term goals of defining the PTP regulatory features and molecular nature, and of developing better drugs for its modulation in vivo. In a series of recent studies, we have shown that the PTP is modulated by electron flux through respiratory complex I (20), which in turn led to the demonstration that the PT is inhibited by Ub₀ and decyl-Ub (21). Inhibition by these quinones could be specifically relieved by Ub₇, which is inactive per se (see Ref. 11 for review). In order to identify the structural features involved in PTP modulation by quinones and to address the issue of mech-

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1 The abbreviations used are: PT, permeability transition; PTP, permeability transition pore; ROS, reactive oxygen species; MOPS, 4-morpholinopropanesulfonic acid; CsA, cyclosporin A; Ub₀, ubiquinone 0; Ub₇, ubiquinone 7; Ub₁₀, ubiquinone 10; TMOH-Ub₇, 2,3,5-trimethyl-6-(3-hydroxyisoamyl)-1,4-benzoquinone; TM-Ub₁₀, 2,3,5-trimethyl-6-geranyl-1,4-benzoquinone; decyl-Ub, decylubiquinone; DM-decyl-Ub, 2,3-dimethyl-6-decyl-1,4-benzoquinone; OH-decyl-Ub, 2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone; DOH-undecyl-Ub, 2,5-dihydroxy-6-undecyl-1,4-benzoquinone; DCFDA, 2,7-dichlorodihydrofluorescein diacetate.
PTP Regulation by Ubiquinone Analogs

Fig. 1. Chemical structure of the ubiquinone analogs used in this study. Groups I, II, and III denote PTP-inhibitory, PTP-activating, and PTP-binding analogs that have no effects per se, respectively.

aniam, we report here an analysis of the effects of quinone structural variants on the PT. We have identified three functional classes, i.e. PTP inhibitory quinones, PTP-inducing quinones, and PTP-inactive quinones, that are nonetheless able to counteract the effects of both inhibitory and inducing quinones. Since the ubiquinone analogs used in this study have a very similar midpoint potential and decrease mitochondrial ROS production to the same extent, these results support the idea that the effects of quinones on the PT are exerted through binding to a common site rather than through redox reactions.

MATERIALS AND METHODS

Rat liver mitochondria were prepared according to standard differential centrifugation procedures in a medium containing 250 mM sucrose, 10 mM Tris-Cl (pH 7.4), and 0.1 mM EGTA-Tris.

Mitochondrial oxygen consumption was measured polarographically at 25 °C using a Clark-type oxygen electrode. Extramitochondrial Ca$^{2+}$ concentration was measured fluorimetrically in the presence of 1 mM Calcium Green-3N exactly as described (20) with excitation and emission wavelengths set at 506 and 521 nm, respectively, with either a PTT Quantamaster C61 or a Kontron SFM 23 spectrofluorometer. Changes of DCFDA fluorescence (8 μM) were determined with the same instruments with excitation and emission wavelengths set at 506 and 521 nm, respectively. Mitochondrial volume changes were measured from the absorbance changes at 540 nm with a Kontron Uvikon 941 spectrophotometer. All instruments were equipped with magnetic stirring and thermostatic control, and the incubation medium is specified in the legend to Fig. 2. Ub0, Ub10, Ub100, decyl-Ub, and CsA were purchased from Sigma (L’Iles d'Abeau, France) while TMOH-Ub5, TM-Ub10, DM-decyl-Ub, OH-decyl-Ub, and DOH-undecyl-Ub were provided by Hoffmann-La Roche (Basel, Switzerland). All other chemicals were of the highest purity commercially available. The chemical structure of the quinones tested in this study is presented in Fig. 1. For the sake of clarity, quinones have been grouped in three classes based on their effects on the PTP: (i) PTP inhibitors (group I), (ii) PTP inducers (group II), and (iii) PTP-inactive analogs that counteract the effects of both group I and II quinones (group III; see “Results” and “Discussion” for details).

RESULTS

Effect of Ubiquinone Analogs on Mitochondrial Ca$^{2+}$ Loading—In the experiments of Fig. 2, energized rat liver mitochondria were loaded with a train of 30 μM Ca$^{2+}$ pulses at 1-min intervals. Although a slight day-to-day variability was observed, mitochondria typically took up and retained Ca$^{2+}$ until the load reached a threshold of about 120 nmol of Ca$^{2+}$ × mg of protein$^{-1}$, a point at which mitochondria underwent a fast process of Ca$^{2+}$ release (trace a). This precipitous Ca$^{2+}$ release was due to opening of the PTP because (i) it was accompanied by depolarization and swelling; and (ii) the Ca$^{2+}$ load required for Ca$^{2+}$ release was dramatically increased by CsA (data not shown, but see Ref. 21). When the quinones listed in Fig. 1 were tested, a striking PTP inhibition was observed with DM-decyl-Ub (trace b), Ub10 (trace c), and TM-Ub10 (trace d). A similar inhibitory effect has been previously reported for Ub0 and decyl-Ub (21), which together with DM-decyl-Ub, Ub10, and TM-Ub10 thus define a class of PTP inhibitory quinones (group I of Fig. 1). Fig. 2 also shows that OH-decyl-Ub (trace e) and DOH-undecyl-Ub (trace f) instead decreased the Ca$^{2+}$ release (data not shown). Finally, the addition of TMOH-Ub5 had no detectable effects on the Ca$^{2+}$ retention capacity (trace g, compare with trace a). As will become clear later, TMOH-Ub5, like Ub5 (21), can remove the effects of group I quinones; these two compounds thus define a third class of PTP-inactive quinones that still demonstrably affect its function (group III of Fig. 1).

The experiments of Fig. 3 report the concentration dependence of the effects of these compounds on the Ca$^{2+}$ retention capacity of rat liver mitochondria. DM-decyl-Ub (panel A) and Ub10 (panel B) increased the Ca$^{2+}$ retention capacity with an optimum of approximately 50 μM, while the effect decreased at higher concentrations without, however, affecting the rate of Ca$^{2+}$ uptake (data not shown). TM-Ub10 also improved the Ca$^{2+}$ retention capacity at concentrations up to 40 μM, but it inhibited the rate of Ca$^{2+}$ uptake at higher concentrations (data not shown). Finally, OH-decyl-Ub (panel D) and DOH-undecyl-Ub (panel E) decreased the Ca$^{2+}$ retention capacity at all concentrations, while TMOH-Ub5 was without any detectable effects (panel F).

Effects of Combinations of Group I and Group III Quinones—In the experiments depicted in Fig. 4, onset of the PT
was monitored from the changes of absorbance at 540 nm, which reflect mitochondrial permeabilization to sucrose. Mitochondria were challenged with a Ca\(^{2+}\) load of 150 nmol \(\times\) mg of protein\(^{-1}\), which caused a detectable PT (traces a in both panels). PTP opening was prevented by TM-Ub10 (panel A, trace b), and this inhibition was relieved by Ub5 (trace c), a group III quinone that does not affect the mitochondrial Ca\(^{2+}\) retention capacity but can remove the inhibitory effects of Ub0 and decyl-Ub but not of CsA (21). Very similar results were obtained when the PTP had been inhibited by other group I quinones (Ub10 and DM-decyl-Ub; results not shown). Thus, Ub5 effectively removed the effects of all available group I quinones (see also Ref. 21).

A similar experiment was performed with the group III quinone TMOH-Ub5 in mitochondria where PTP opening had been inhibited with decyl-Ub (Fig. 4, trace b). It can be seen that TMOH-Ub5 relieved the inhibitory effects of decyl-Ub (trace c) but not of CsA (results not shown). A similar effect of TMOH-Ub5 was observed in mitochondria where the PTP had been inhibited by Ub5 but not by the other group I quinones Ub0 and TM-Ub10 (results not shown). These data suggest that group III quinones may bind to the same site as group I quinones, and that the final effect on the PTP may depend on their relative affinities for the PTP regulatory site(s).

Effects of Combinations of Group II and Group III Quinones—In the experiments depicted in Fig. 5, we tested whether group III quinones can prevent the effects of the PTP-inducing group II quinones. In the experiments of panel A, rat liver mitochondria were challenged with a Ca\(^{2+}\) load of 60 nmol \(\times\) mg of protein\(^{-1}\), which is not sufficient for PTP opening under these experimental conditions (trace a). In the presence of OH-decyl-Ub, PTP opening readily followed the addition of the same Ca\(^{2+}\) load (trace b), and this inducing effect on the PTP was prevented by Ub5 (trace c). The experiments of panel B show that neither OH-decyl-Ub alone (trace b) nor the combination of OH-decyl-Ub and Ub5 (trace c) affected the rate of Ca\(^{2+}\) uptake prior to onset of the PT relative to untreated mitochondria (trace a), a finding that was confirmed in experiments with an appropriate time scale (omitted for clarity). It is noteworthy that the combination of OH-decyl-Ub and Ub5 was unable to fully restore the Ca\(^{2+}\) retention capacity of untreated mitochondria (compare traces c and a).

Similar experiments were carried out with different combinations of group II and III inhibitors. We found that, at vari-

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**FIG. 2.** Effect of quinones on the Ca\(^{2+}\) retention capacity of rat liver mitochondria. The incubation medium contained 250 mM sucrose, 1 mM P-Tris, 10 mM Tris-MOPS, 5 mM glutamate-Tris, 2.5 mM malate-Tris, 1 mM Calcium Green-5N. The final volume was 2 ml, pH 7.4, 25 °C. Experiments were started by the addition of 2 mg of mitochondria, followed by 60 μM DM-decyl-Ub (trace b), 50 μM Ub10 (trace c), 50 μM TM-Ub10 (trace d), 50 μM OH-decyl-Ub (trace e), 25 μM DOH-undecyl-Ub (trace f), or 50 μM TMOH-Ub5 (trace g) (additions not shown). In trace a, no quinones were added. Each arrow indicates the addition of one 30 μM Ca\(^{2+}\) pulse.

**FIG. 3.** Ca\(^{2+}\) retention capacity of rat liver mitochondria at increasing concentrations of ubiquinone analogs. Experimental conditions were the same as in Fig. 2 in the presence of the indicated concentration of DM-decyl-Ub (panel A), Ub10 (panel B), TM-Ub10 (panel C), OH-decyl-Ub (panel D), DOH-undecyl-Ub (panel E), and TMOH-Ub5 (panel F). Trains of 30 μM Ca\(^{2+}\) pulses were added at 1-min intervals exactly as shown in Fig. 2. Values on the ordinate denote the Ca\(^{2+}\) retention capacity as the amount taken up prior to the Ca\(^{2+}\) addition causing Ca\(^{2+}\) release.
ance from Ub₅, TMOH-Ub₅ was unable to prevent the inducing effects of OH-decyl-Ub, and that neither Ub₁₀ nor TMOH-Ub₅ could prevent the PTP-inducing effects of DOH-undecyl-Ub (results not shown).

Effects of Combinations of Group I and Group II Quinones—We next tested whether the group II, PTP-inducing quinones could contrast the effects of the group I, PTP-inhibiting quinones in assays based on the mitochondrial Ca²⁺ retention capacity. Fig. 6 shows that (i) OH-decyl-Ub was able to counteract the increased Ca²⁺ retention capacity elicited by Ub₁₀ (trace c, compare with trace b), yielding a result that came close to that of untreated mitochondria (trace a), and (ii) that DOH-undecyl-Ub likewise counteracted the large increase of Ca²⁺ retention caused by Ub₀, the most efficacious PTP inhibitor we found so far (trace e, compare with trace d). Similar results were obtained when mitochondria were treated with the other group I quinones decyl-Ub, DM-decyl-Ub, or TM-Ub₁₀ (data not shown). These experiments demonstrate that group II quinones can counteract the effects of all group I quinones, further suggesting an interaction at a common site.

Effects of Ubiquinone Analogs on Respiration and ROS Production—We have previously shown that there is no obvious correlation between the effects of quinones on respiration and on the PTP. Thus, both the group I Ub₀ and the group III Ub₅ inhibited uncoupled respiration, whereas the group I decyl-Ub had only marginal effects (21). We extended this analysis to the quinones tested in this study. Since PTP opening is accompanied by loss of NADH, which in turn causes respiratory inhibition with complex I substrates (20, 22), these experiments were performed in the presence of CsA. Fig. 7A shows that the group I inhibitory quinones DM-decyl-Ub and Ub₁₀ (traces b) or 50 µM OH-decyl-Ub plus 200 µM Ub₀ (traces c). Where indicated, the addition of Ca²⁺ was 60 µM in panel A or of trains of 25 µM Ca²⁺ pulses in panel B.
drial production of ROS as assessed by the fluorescence changes of DCFDA. Fig. 8 shows that addition of DCFDA to respiring mitochondria led to a measurable fluorescence increase (trace a), which was slowed down by group I Ub (trace b), group II OH-decyl-Ub (trace c), and group III Ub5 (trace d). Similar effects were obtained with all the quinone derivatives described in this study (omitted for clarity). Thus, no obvious correlation exists between mitochondrial ROS production and effects of quinones on the PTP.

**DISCUSSION**

In this paper we have shown that ubiquinone analogs profoundly affect the mitochondrial PT. We have identified three classes of quinones: (i) group I, or PTP inhibitory quinones (Ub10, TM-Ub10, and DM-decyl-Ub), which mimic the effects we reported previously for Ub0 and decyl-Ub (21); (ii) group II, or PTP-inducing quinones (OH-decyl-Ub and DOH-undecyl-Ub), which dramatically decrease the Ca2+ load required for PTP opening and represent a previously unrecognized functional class of PTP inducers; and (iii) group III, or PTP-inactive quinones (TMOH-Ub5 (this paper) and Ub5 (Ref. (21)), which are nonetheless able to counteract the effects of group I and II quinones.

**Structure-Function Correlation**—An interesting issue is represented by the structure-function correlation for the specific effects of quinone analogs on the PTP. Inspection of the structures (Fig. 1), as well as comparison with the results of a previous study (21), suggests that seemingly minor changes can profoundly affect the quinone interactions with the PTP. Thus, (i) a methyl group in position 5 of the benzoquinone ring is not required for PTP inhibition since DM-decyl-Ub is active, (ii) the radical in position 6 affects both the potency and the quality of the effects of quinones on the PTP, and (iii) a methoxy or a methyl group in positions 2 and 3 appears to be required for PTP inhibition (group I) or for displacement (group III) because 1,4-benzoquinone and 2-methoxy-1,4-benzoquinone are not effective (21). Several problems must be kept in mind, however, when these complex results are analyzed. Although there is no direct correlation between the effects of quinones on the PTP and on respiration, early respiratory inhibition may preclude the analysis of potential PTP inhibitory quinones in protocols based on energy-dependent Ca2+ uptake, thus limiting the range of compounds that can be tested. Also, it should be noted that a lack of demonstrable effects of quinones does not necessarily mean that they are not
interacting with the PTP. Since PTP-inactive, group III quinones can only be recognized by competition experiments, quinones binding the PTP with low affinity could escape detection. Finally, we have not been able to conclusively address the issue of whether the interactions of quinones of the three groups is competitive or non-competitive in nature. The apparent EC\textsubscript{50} values obtained in this type of experiments were not entirely reliable either because of the biphasic nature of the response, and/or because increasing concentrations of quinones caused inhibition of Ca\textsuperscript{2+} uptake. We therefore consider the present results as the starting point for more detailed mechanistic studies that will be addressed through the synthesis and the analysis of further structural variants.

**Mechanistic Aspects**—A key issue is the mechanism through which quinones modulate the PTP. The analogs used in this study have similar midpoint potentials, and this makes a comparison of their effects on the PTP and on ROS production, which rather appears to be decreased by all quinones tested in this study (Fig. 8). In principle, the existence of inducing and inhibiting quinones could be explained by the presence of two quinone binding sites, one responsible for inhibition and one for activation. Assuming that group I quinones may also bind to the latter site with lower affinity, the biphasic response observed with some quinones could be easily explained because high concentrations of group I quinones caused inhibition of Ca\textsuperscript{2+} uptake. We therefore consider the present results as the starting point for more detailed mechanistic studies that will be addressed through the synthesis and the analysis of further structural variants.

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