

A 22 kDa polyanion inhibits carnitine-dependent fatty acid oxidation in rat liver mitochondria

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Abstract The transport of activated fatty acids across the mitochondrial outer membrane has not been fully addressed. A polyanion ($M_n = 22$ kDa) inhibited the ADP-stimulated carnitine-dependent oxidation of both palmitoyl-CoA and palmitate plus CoA as well as mitochondrial hexokinase binding. In contrast, the oxidation of palmitoylcarnitine plus malate, as well as glutamate oxidation, was essentially unaffected. Mitochondrial carnitine palmitoyltransferase-1 was not inhibited by the polyanion. The data suggest an additional component in carnitine-dependent mitochondrial fatty acid oxidation, possibly porin.

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Key words: Mitochondrion; Carnitine; Fatty acid oxidation; Porin; Polyanion; Carnitine palmitoyltransferase

1. Introduction

Recent evidence has shown that the mitochondrial outer membrane plays an important role in regulating metabolite flux, much of which is controlled by a voltage-dependent anion channel (VDAC), also known as mitochondrial porin [1–6]. Each mitochondrial outer membrane channel is thought to be formed by a monomeric VDAC polypeptide with a molecular weight in the range of 30 kDa. In the open state, the pore radius of VDAC is estimated to be 1.2–1.5 nm, large enough to allow for passage of the non-electrolytes polyethylene glycol ($M_{av} = 3.4$ kDa), inulin ($M_{av} = 5.0$ kDa) and dextran ($M_{av} = 6.0$ kDa). In the closed state, it is impermeable to inulin and PEG-3.4 kDa and the estimated pore size is 0.85 nm.

Regulation of porin in its isolated form in planar phospholipid bilayer or liposome reconstitution experiments and in its native environment using patch-clamp techniques has been demonstrated [7–17]. Collectively, the data suggest that porin plays a role in metabolite flux by modulation of its gating properties. Additionally, a protein modulating the open/closed state of the pore has been identified, partially purified and characterized from several sources [12,17]. This protein modulator was shown to control respiration, most likely by limiting the ADP and substrate supply through VDAC, thereby providing a means for regulating the mitochondrial function. Porin has also been shown to interact with other proteins

present in contact sites, for example hexokinase [18–21]. This specific binding of hexokinase to VDAC at contact sites allows for preferential access to mitochondrial-produced ATP and may also contribute to the regulation of mitochondrial energy metabolism.

A number of studies on the function of VDAC in regulating the metabolite flux have involved the use of large polyvalent anions [13,16]. One, a synthetic polyanion prepared by König et al. (a copolymer of methacrylate, maleate and styrene in a 1:2:3 proportion), strongly inhibited inner membrane transporters and ATP/ADP uptake in intact mitochondria [22–27]. The polyanion at nM concentrations was shown to induce a closed state of the mitochondrial outer membrane channel. Since the polyanion is too large to enter through the pore, polyanion-induced closure of VDAC is thought to be responsible for the observed inhibition of the inner membrane transporters and ATP/ADP uptake.

While the transport of negatively charged metabolites across the mitochondrial outer membrane through VDAC has been well characterized, the transport of activated fatty acids has not been addressed. We proposed that the transport of the negatively charged CoA derivatives of fatty acids through the mitochondrial outer membrane also involves VDAC. Closure of the outer membrane VDAC would not only limit ADP and ATP transport, but would also limit the transport of fatty acyl-CoAs into the mitochondria. In order to test this hypothesis, a polyanion similar to König's was prepared and used in experiments with isolated rat liver mitochondria.

2. Materials and methods

2.1. Synthesis of polyanion

All reagents for the synthesis of polyanion were obtained from Aldrich Chemical Company and used as received, except where noted. Methacrylic acid was distilled under reduced pressure. Styrene was washed with 10% NaOH, five times with distilled water, dried with $MgSO_4$, filtered and passed through a bed of neutral alumina. Toluene was washed with concentrated H_2SO_4 , five times with distilled water, dried with $MgSO_4$ and filtered. All solutions were purged with argon prior to use. The molecular weight was determined on a Perkin-Elmer Series 10-LC GPC/HPLC instrument using tetrahydrofuran (THF) as solvent and polystyrene standards for calibration. 1H -NMR (200 MHz) measurements were obtained on a Varian Gemini 200 NMR spectrometer using acetone- d_6 as solvent and tetramethylsilane as reference.

Polyanion was prepared following the procedure outlined by König et al. [22] with slight modifications. The initiator used for polymerization was 2,2-azobisisobutyronitrile (AIBN) and the reaction time was reduced to 10 min. An average molecular weight (M_n) of 22 kDa and polydispersity index (M_w/M_n) of 2.3 versus polystyrene standards was obtained by size exclusion chromatography. The copolymer composition determined by 1H -NMR (acetone- d_6) was 48% styrene, 37.5% maleate and 14.5% methacrylate (3.3:2.6:1); δ (ppm) 0.9 (m, methacrylate $-CH_3$), 2.3 (m, methacrylate $-C-CH_2-$ and styrene $-CH-$

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Abbreviations: VDAC, voltage-dependent anion channel (porin); CPT-I, malonyl-CoA sensitive carnitine palmitoyltransferase; PA-22, 22 kDa polyanion

CH₂-), 2.5 (m, styrene -CH-CH₂-), 3.3 (m, maleate -CH-CH-), 7.25 (m, styrene aromatic H).

While we were initially unable to obtain the original 10 kDa polyanion from Dr T. König, at the completion of this study, we received a small sample of König's polyanion from Dr M. Colombini.

2.2. Isolation of rat liver mitochondria

Mitochondria were isolated from livers of male Sprague-Dawley rats (390–760 g) in 220 mM mannitol, 70 mM sucrose, 5 mM MOPS, pH 7.4 (MSM), by differential centrifugation according to Hoppel et al. [28]. Mitochondria used for hexokinase binding experiments were further purified using a self forming Percoll gradient [28].

2.3. Assays

Oxygen uptake was monitored at 30°C using a Clark oxygen electrode as described by Tomec and Hoppel [29]. The incubation medium contained 100 mM KCl, 50 mM MOPS, 5 mM KPi, 1.0 mM EGTA, 1.0 mg defatted bovine serum albumin (BSA)/ml, pH 7.0, and 1.0 mg of mitochondrial protein in a final volume of 0.5 ml. Respiration was determined in the absence and presence of polyanion, 0–9.1 nmol polyanion per mg mitochondrial protein, by the addition of substrate to the incubation medium following depletion of endogenous substrates by small amounts of ADP. Experiments with mitochondria subjected to digitonin treatment or hypotonic treatment to disrupt the outer membrane were performed as described by Bodrova et al. [30]. Incubation medium contained 250 mM sucrose (45 mM sucrose for hypotonic conditions), 1 mM EGTA, 5 mM MOPS, 5 mM KPi, pH 7.4, and 1.0 mg rat liver mitochondria. Since these experiments were done with palmitoyl-CoA as the substrate, 1 mg defatted BSA/ml was added to the incubation media. Disruption of outer membrane with 0.1 mM digitonin was done in the 250 mM sucrose medium. The digitonin concentration for outer membrane disruption was determined by titration experiments and found to be similar to that used by Bodrova et al. [30]. Integrity of the mitochondrial outer membrane was assessed as described by Xu et al. [31].

Hexokinase was isolated as described by Wilson et al. [32]. For determination of hexokinase binding, rat liver mitochondria (2.5 mg) were incubated on ice for 30 min in a final volume of 250 µl in MSM, with the following additions: (1) no added polyanion or hexokinase, (2) 11.3 nmol polyanion, (3) 500 mU hexokinase, (4) 11.3 nmol polyanion and 500 mU hexokinase. After 30 min incubation at 0°C, the samples were layered on 1 ml MSM and the mitochondria recovered by centrifugation (Beckman microcentrifuge, 12000 × g, 5 min, 4°C). The pellets were resuspended in 250 µl MSM, 1% cholate and 50 µl aliquots (0.5 mg) were assayed for hexokinase activity [33]. Aliquots of samples 1 and 2 were also assayed following the addition of 10 mU hexokinase per mg mitochondrial protein.

Carnitine palmitoyltransferase I (CPT-I) activity was measured using the radiochemical forward assay exactly as referenced [28]. CPT-I activity is defined as the malonyl-CoA/etomoxiryl-CoA suppressible activity. Etomoxiryl-CoA was synthesized as described by Hoppel et al. [28]. Both hexokinase and CPT-I activities are expressed as nmol/min or mU/mg mitochondrial protein.

Protein was determined with the biuret assay using BSA as a standard [34].

3. Results

3.1. Characterization of polyanion

Polymerization, carried out in the presence of AIBN as the initiator, led to a polyanion with a slightly different composition than the 1:2:3 methacrylate:maleate:styrene obtained by König et al. The maleate content, based on ¹H-NMR data, is 37.5%, which is slightly higher than that obtained by König. The styrene content is 48% and the methacrylate content is 14.5%. The styrene and methacrylate content is slightly lower than that found in König's polyanion. Size exclusion chromatography of the polyanion versus polystyrene standards yielded a molecular weight (*M_n*) of 22 kDa and polydispersity (*M_w*/*M_n*) of 2.3, compared to a molecular weight of 10 kDa obtained by König. The results show that the polyanion synthesized by us has a high molecular weight, making it too

large to pass through the mitochondrial outer membrane pores, and a fairly narrow molecular weight distribution, indicating little or no contamination by low molecular weight polymerization products. To avoid confusion with König's polyanion, we will label this polyanion as 22 kDa polyanion (PA-22).

3.2. Effect of polyanion (22 kDa) on mitochondrial respiration

We proposed that the polyanion would not only affect adenine nucleotide movement through porin, but also that of palmitoyl-CoA. The influence of polyanion (PA-22) on glutamate oxidation was examined (Fig. 1). The state 3 (ADP-available) rate was not affected. Additionally, no effect was observed on the state 4 (ADP-limiting) rate, respiratory control ratio and ADP/O ratios or when either high ADP or uncoupler (DNP) were used. These data contrast with the observation of König et al. of a strong, time-dependent inhibition of the above respiratory parameters with glutamate plus malate as a substrate [22]. Pyruvate and octanoate oxidation (data not shown) also are not affected.

Next, we tested the effect of PA-22 on fatty acid oxidation in isolated rat liver mitochondria. As shown in Fig. 2, the oxidation of palmitoylcarnitine in the presence of malate was little affected by PA-22. The state 3, high ADP and uncoupled respiratory rates were slightly decreased at the amount of PA-22 used (4.5 nmol/mg mitochondrial protein). In contrast, the carnitine-dependent oxidation of palmitoyl-CoA in the presence of malate is inhibited by PA-22. At 4.5 nmol PA-22/mg mitochondrial protein, the state 3 rate

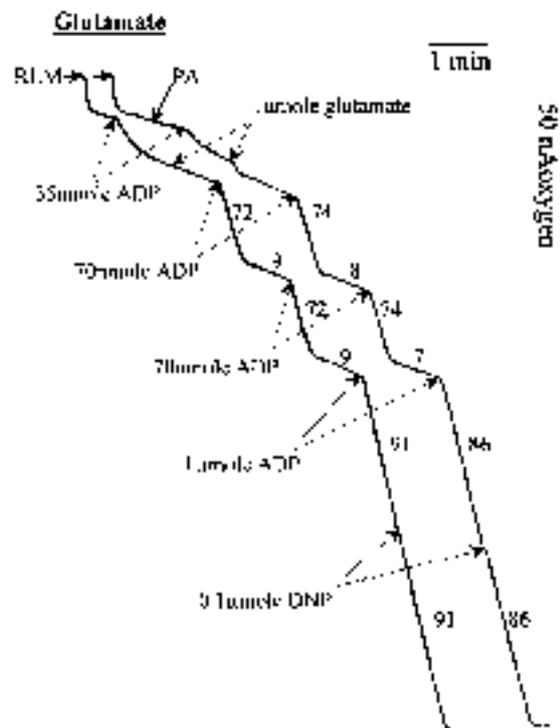


Fig. 1. Representative oxidative phosphorylation traces with glutamate as substrate in the absence and presence of polyanion. Additions of substrates were made following initial addition of 1 mg rat liver mitochondria (RLM) and 4.5 nmol polyanion (PA; when indicated) to 0.5 ml oxygen electrode mixture. The numbers on the tracings represent the rate of oxygen consumption in nanoatoms oxygen/min/mg mitochondrial protein.

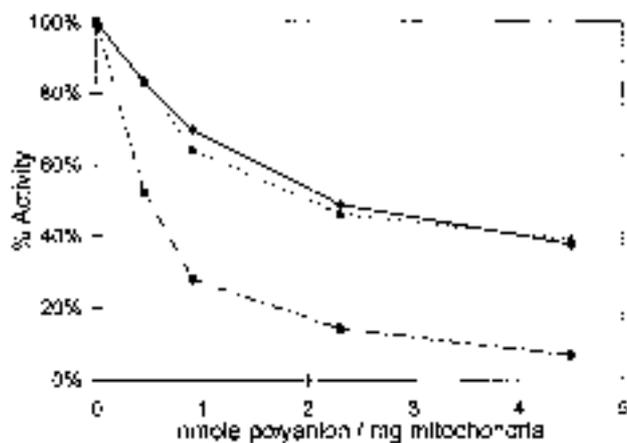


Fig. 4. Effect of polyanion on maximal ADP-stimulated oxidative phosphorylation of palmitoyl-CoA in intact and disrupted rat liver mitochondria. Rates (nanoatoms oxygen/min/mg) obtained at 0 nmol polyanion were set to 100% for each experiment: 250 mM sucrose (◆)=57.4, 250 mM sucrose, 0.1 mM digitonin (▲)=47.3, 45 mM sucrose (●)=48.8.

3.3. Effect of polyanion on hexokinase binding to rat liver mitochondria

The polyanion prepared by König et al. has been reported to bind to porin (also known as hexokinase binding protein) with concomitant changes in the open/closed state of this outer membrane pore protein. Does PA-22 interact with porin? To address this question, we utilized the specific binding of hexokinase to porin and tested the binding of hexokinase to isolated rat liver mitochondria in the presence and absence of polyanion. Hexokinase binding in rat liver mitochondria (37.8 mU hexokinase/mg mitochondria) was reduced over 80% in the presence of 4.5 nmol polyanion per mg mitochondrial protein (6.5 mU hexokinase/mg mitochondria). When equal amounts of hexokinase were added to the assay mixtures containing either untreated mitochondria or mitochondria that were exposed to PA-22, complete recovery of hexokinase activity was observed. Thus, there was no effect of residual or bound polyanion on the measurement of hexokinase activity. These data suggest that PA-22 interacts with porin.

3.4. Effect of polyanion on CPT-I activity

Since the polyanion inhibited the carnitine-dependent oxidation of palmitate and palmitoyl-CoA, which is reliant on CPT-I, but not the oxidation of palmitoylcarnitine, we asked whether PA-22 inhibits the formation of palmitoylcarnitine. To this end, we determined the maximally expressed activity of CPT-I in isolated rat liver mitochondria and used both malonyl-CoA as well as etomoxiryl-CoA as specific inhibitors to delineate CPT-I (Table 1). As evident from the near complete inhibition by malonyl-CoA and etomoxiryl-CoA, at both 50 and 160 μ M palmitoyl-CoA concentrations, all overt activity measured is CPT-I in these mitochondria. Under these conditions, PA-22 did not inhibit CPT-I activity.

4. Discussion

We have synthesized a polyanion with a molecular weight of 22 kDa which selectively inhibits the carnitine-dependent oxidation of palmitate and palmitoyl-CoA. The overall rate of oxygen consumption is inhibited by approximately 60%. How-

ever, as state 4 rates are not affected, the percentage inhibition of substrate-supported state 3 rates by PA-22 is nearly complete. Since the oxidation of palmitoylcarnitine is not inhibited at PA-22 concentrations where the carnitine-dependent oxidation of palmitate and palmitoyl-CoA is already maximally inhibited, the site of inhibition must be between palmitoyl-CoA and palmitoylcarnitine. A potential step could be CPT-I, which catalyzes the formation of palmitoylcarnitine from palmitoyl-CoA and carnitine. Direct measurement of maximally expressed CPT-I activity under conditions where virtually all activity was suppressed by malonyl-CoA, as well as etomoxiryl-CoA, revealed no inhibition by PA-22. These data rule out a direct interaction between CPT-I and PA-22 and eliminate CPT-I as the site of inhibition of fatty acid oxidation by PA-22. Thus, a step affecting palmitoyl-CoA but not the formation or the handling of palmitoylcarnitine is implicated.

Since porin is known to be responsible for metabolite flux across the mitochondrial outer membrane, we raised the question whether PA-22 limits the availability of palmitoyl-CoA to the catalytic site of CPT-I by interacting with the outer membrane pore channel protein porin? Similar to König's polyanion, which has been shown to limit substrate supply through porin, the PA-22 was shown to interact with porin, however, the metabolic consequences of the binding of these two polyanions are different. While König's polyanion inhibited state 3 respiration with glutamate/malate and succinate (and in preliminary experiments in our laboratory, it also inhibited the carnitine-dependent oxidation of palmitoyl-CoA), the PA-22 selectively inhibited the carnitine-dependent oxidation of palmitoyl-CoA and palmitate plus CoA, but had no effect on the other substrates we tested (glutamate, palmitoylcarnitine, pyruvate, octanoate). Furthermore, while inhibition of glutamate oxidation by König's polyanion was reversible by high ADP and uncoupler, the inhibition of the carnitine-dependent oxidation of palmitoyl-CoA by both polyanions was not reversed. Thus, the inhibition of carnitine-dependent fatty acid oxidation by PA-22 must be through a mechanism other than the inhibition of adenine nucleotide transport through the mitochondrial outer membrane.

In experiments involving the carnitine-dependent palmitoyl-CoA oxidation in mitochondria that had the outer membrane damaged by either digitonin treatment, hypotonic swelling failed to reverse inhibition by PA-22. In preliminary experiments with French press mitoplasts, in which 81–85% of the outer membrane marker activity was removed, 50% of CPT-I and long-chain acyl-CoA synthetase (LCAS) activity remained and there was no loss of carnitine-dependent palmitoyl-CoA oxidation [28], inhibition by PA-22 was still observed. The CPT-I and LCAS activities remaining with these

Table 1
Effect of polyanion on CPT-I activity

Incubation conditions	[Palmitoyl-CoA]	
	50 μ M	160 μ M
No addition	17.9	29.6
Etomoxiryl-CoA ^a	0.4	1.2
200 μ M Malonyl-CoA	1.1	3.7
Polyanion (4.5 nmol/mg mitochondrial protein)	17.8	34.6

^aMitochondria were pre-incubated with 0.5 μ M etomoxiryl-CoA as described in [28].

French press-generated mitoplasts are localized in contact sites [28]. These mitochondrial structures are also present in mitochondria subjected to digitonin treatment [18] or hypotonic swelling [19] and it are these contact sites that represent the purported site of action for PA-22.

Since the size of the polyanion is too large to cross the mitochondrial outer membrane, its inhibitory effect on palmitate/palmitoyl-CoA oxidation must be exerted at the cytosolic side of the outer membrane. With the catalytic site facing the intermembrane space and the regulatory or malonyl-CoA binding site facing the cytosol [35], the inhibition of palmitoyl-CoA oxidation by the polyanion could be envisaged as preventing the movement of palmitoyl-CoA through porin from the cytosol to the catalytic site of CPT-I on the inner surface of the mitochondrial outer membrane. The fact that the polyanion strongly inhibited the specific binding of hexokinase to porin in isolated rat liver mitochondria, combined with a lack of CPT-I inhibition, lends support to this possibility. Recently, both the catalytic and malonyl-CoA binding site of CPT-I were reported to be localized to the cytosolic side of the mitochondrial outer membrane [36]. Again, the lack of inhibition of maximally expressed CPT-I activity suggests a mode of interaction of PA-22 on palmitoyl-CoA oxidation that does not involve CPT-I.

In conclusion, the PA-22 selectively inhibits the carnitine-dependent oxidation of palmitoyl-CoA. The data suggest that this inhibition is accomplished by preventing the provision of palmitoyl-CoA to CPT-I. The peripheral benzodiazepine receptor, which consists of porin (outer membrane), adenine nucleotide translocase (inner membrane) and an 18 kDa outer membrane protein, is involved in cholesterol transport through the mitochondrial outer membrane. Binding of the diazepam binding inhibitor (DBI; 10 kDa) greatly facilitates the transport of cholesterol through the mitochondrial outer membrane to the cytochrome P450 side chain cleavage enzyme localized on the outer surface of the adrenal mitochondrial inner membrane [37,38]. By analogy, we speculate that a similar mechanism exists for the movement of long-chain acyl-CoAs through the mitochondrial outer membrane. The near complete homology between the DBI and the acyl-CoA binding protein (ACBP) certainly enhances this possibility. Binding of the polyanion to porin could affect the transport of long-chain acyl-CoAs either indirectly by preventing the interaction of ACBP with porin or directly by affecting pore closure. Further studies concerning the transport of long-chain acyl-CoAs through the mitochondrial outer membrane are warranted.

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