Biochimica et Biophysica Acta 1827 (2013) 1156–1164

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Q-site inhibitor induced ROS production of mitochondrial complex II is attenuated by TCA cycle dicarboxylates

Ilka Siebels, Stefan Dröse *

Clinic of Anesthesiology, Intensive-Care Medicine and Pain Therapy, Goethe-University Hospital, Theodor-Stern Kai 7, Frankfurt am Main, Germany

ARTICLE INFO

Article history:
Received 13 May 2013
Received in revised form 12 June 2013
Accepted 14 June 2013
Available online 22 June 2013

Keywords:
Mitochondria
Complex II
Succinate:ubiquinone oxidoreductase
Reactive oxygen species (ROS)
Dicarboxylates
Atpenin A5

ABSTRACT

The impact of complex II (succinate:ubiquinone oxidoreductase) on the mitochondrial production of reactive oxygen species (ROS) has been underestimated for a long time. However, recent studies with intact mitochondria revealed that complex II can be a significant source of ROS. Using submitochondrial particles from bovine heart mitochondria as a system that allows the precise setting of substrate concentrations we could show that mammalian complex II produces ROS at subsaturating succinate concentrations in the presence of Q-site inhibitors like atpenin A5 or when a further downstream block of the respiratory chain occurred. Upon inhibition of the ubiquinone reductase activity, complex II produced about 75% hydrogen peroxide and 25% superoxide. ROS generation was attenuated by all dicarboxylates that are known to bind competitively to the substrate binding site of complex II, suggesting that the oxygen radicals are mainly generated by the unoccupied flavin site. Importantly, the ROS production induced by the Q-site inhibitor atpenin A5 was largely unaffected by the redox state of the Q pool and the activity of other respiratory chain complexes. Hence, complex II has to be considered as an independent source of mitochondrial ROS in physiology and pathophysiology.

1. Introduction

The mitochondrial respiratory chain not only provides the main portion of ATP in mammalian cells, but also is a major source of reactive oxygen species (ROS) [1–3]. While an increased mitochondrial ROS production has been mainly associated with several pathophysiological conditions, it is increasingly evident that ROS produced under physiological conditions can also serve as essential signaling molecules [4,5]. Within the respiratory chain, complexes I (NADH:ubiquinone oxidoreductase) and III (cytochrome bc_1 complex; ubiquinol:cytochrome *c* oxidoreductase) are generally regarded as the main generators of ROS [1–3,6], whereas a contribution of complex II seemed to be negligible. On the other hand, it has been recognized that complex II activity can modulate the ROS production of complexes I and III under specific conditions (overview in [7]). A high succinate concentration/complex II activity is the prerequisite to induce superoxide production at complex I due to reverse electron transfer (RET) [8–12]. Additionally, complex II activity can influence the antimycin A induced superoxide production at the Q_o site of complex III [13,14] that is maximal at intermediate redox-states of the Q-pool [15,16].

Several studies have shown that mammalian complex II produces hardly any ROS at saturating succinate concentrations in isolated mitochondria or submitochondrial particles (SMP) [13,17,18]. However, a malfunction of complex II has been associated with different pathophysiological conditions including specific forms of cancer and it seems plausible that disease-associated mutations increase the mitochondrial ROS production [19–23]. Otherwise, complex II Q-site inhibitors have been used to specifically induce cell death of cancer cells which also involves the production of ROS (reviewed in [24,25]). Hence, understanding the complicated intertwining connection between complex II and mitochondrial ROS production is essential to clarify its role in physiology and pathophysiology [7].

The primary physiological function of complex II is to oxidize succinate to fumarate and transfer the electrons derived from this reactions onto ubiquinone that fuels the downstream respiratory chain complexes III and IV [26]. This reaction sequence creates a direct link between two major energy-converting mitochondrial pathways, the tricarboxylic acid cycle (TCA or Krebs cycle) and the respiratory chain. The catalytic mechanism and the structural organization of complex II are well known [26,27]. The succinate oxidation takes place in the dicarboxylate binding site of the matrix-facing SdhA-subunit and involves a covalently bound FAD. The electrons are transferred via three iron–sulfur clusters (located in SdhB) to the (proximal) ubiquinone binding (Q-site) site located at the interface between SdhB and the two membrane-bound subunits SdhC and SdhD. The two membrane-bound subunits additionally bind

Abbreviations: DQA, 2-n-decyl-quinazolin-4-yl-amine; FAD, flavin adenine dinucleotide; HRP, horseradish peroxidase; NAD^+/NADH, oxidized/reduced from of nicotinamide adenine dinucleotide; Q, ubiquinone; QFR, quinol:fumarate oxidoreductases; Q-site, ubiquinone reduction site of complex II; Q_o site, ubiquinol oxidation site of cytochrome bc_1 complex (complex III); RET, reverse electron transfer; ROS, reactive oxygen species; SMP, submitochondrial particles; SOD, superoxide dismutase; SQR, succinate:quinone oxidoreductases; TTFA, 2-thenoyltrifluoroacetone

* Corresponding author. Tel.: +49 69 6301 87824; fax: +49 69 6301 87822.
E-mail address: Stefan.Droese@kgu.de (S. Dröse).

0005-2728/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbabio.2013.06.005

a heme *b* that is located off-pathway from the electron transferring cofactors and does not contribute to the electron transfer between the two substrate binding sites [27].

It has been shown that the structurally and functionally related bacterial succinate:quinone oxidoreductases (SQR) (overview in [19,26,27]) and especially the quinol:fumarate oxidoreductases (QFR) can be a major source of ROS under aerobic conditions [28,29]. In a detailed analysis, Messner and Imlay [29] observed a bell-shaped dependence of the succinate-fueled ROS-production by SOR and QFR from *Escherichia coli* that peaked at a substrate concentration around 1 mM. While the *E. coli* SQR produced only superoxide, the QFR produced a mixture of superoxide and hydrogen peroxide [29]. Recently, Quinlan and colleagues could show that also the mammalian complex II can produce ROS at significant rates if the downstream respiratory chain is blocked [30]. Maximal ROS production occurred in isolated skeletal muscle mitochondria at concentrations of ~400 μM succinate while a saturating concentration largely suppressed the ROS release [30]. The authors concluded that the ROS generation occurs at the flavin in the unoccupied dicarboxylate binding site (also called site II_F).

In the present investigation, we used submitochondrial particles derived from bovine heart mitochondria to investigate the ROS generation of complex II in more detail. We confirmed that complex II produces ROS at subsaturating succinate concentrations in the presence of Q-site inhibitors like atpenin A5 or when a further downstream block of the respiratory chain occurred. Upon inhibition of the ubiquinone reductase activity, complex II mainly produced hydrogen peroxide. The ROS production was reduced not only by higher succinate concentrations, but also by other TCA cycle intermediates that are known to bind competitively to the dicarboxylate binding site. Finally, the atpenin A5 induced ROS production was largely unaffected by the activity of other respiratory chain complexes which indicates that complex II is an independently inducible mitochondrial ROS generator.

2. Material and methods

2.1. Materials

DQA (2-n-decyl-quinazolin-4-yl-amine, SAN 549) was a generous gift from Prof. Ulrich Brandt (Radboud University Nijmegen, The Netherlands), atpenin A5 was from Enzo Life Sciences (Lörrach, Germany). Since diluted atpenin A5 solutions have a limited stability, they were freshly prepared from a 1 mM stock before starting the experiments. Ampliflu Red™ (10-acetyl-3,7-dihydroxyphenoxazine), HRP, SOD (from bovine liver), antimycin A, stigmatellin, diazoxide, TTFA and all other chemicals were from Sigma-Aldrich (Steinheim, Germany).

2.2. Preparation of SMP

Submitochondrial particles from bovine heart mitochondria were prepared as previously described [31]. The preparation that was used throughout this investigation had a protein concentration of 32.0 mg/ml (29.8 μM haem *b* and 37.5 μM haem aa_3).

2.3. Measurements of superoxide and H_2O_2 production

The production of reactive oxygen species (superoxide and hydrogen peroxide) was generally measured in a Spectra Max Plus^{384} microplate reader (Molecular Devices) at 37 °C by absorbance with the exception of the data presented in Fig. 1B that were measured in a Spectra Max M2^e Multi Mode Reader (Molecular Devices). SMP (24 μg protein per cavity; total assay volume 200 μl) were dissolved in a buffer containing 75 mM sodium phosphate, 1 mM EDTA, and 1 mM $MgCl_2$ (pH 7.2) supplemented with 400 U ml^{-1} SOD. In some experiments, the pH was raised to 8.0. SOD supplementation was necessary to ensure the complete conversion of superoxide to hydrogen peroxide (SOD increased the detected rate of H_2O_2 production by ~20%) and to reduce the NADH-dependent background reaction of HRP with 10-acetyl-3,7-dihydroxyphenoxazine [32,33]. The respiratory chain of the SMP was usually fueled with different succinate concentrations (0.01–5.0 mM) and/or 250 μM NADH. Background rates were determined in the absence of SMP. In general, triplicates of all data points were acquired and the mean value and standard deviation are shown. Respiratory chain inhibitors (complex I: DQA; complex II: Q-site — atpenin A5, dicarboxylate site — malonate; complex III Q_o site: stigmatellin) and different TCA cycle intermediates (fumarate; oxaloacetate; malate; citrate) were applied as indicated. The hydrogen peroxide production was measured by the HRP (0.1 U ml^{-1}) catalyzed conversion of 50 μM 10-acetyl-3,7-dihydroxyphenoxazine (Amplifu Red™, also called Amplex Red®) to resorufin [34] that can be followed at the absorption maxima of 571 nm ($\varepsilon_{571\ nm}$: 54 mM^{-1} cm^{-1}) [33]. Kinetic data were usually recorded for 20 min at an interval of 13 readings min^{-1}. The superoxide production was monitored as the SOD-sensitive reduction of acetylated cytochrome *c* [35] at Δ550–540 nm, i.e. the reduction of 25 μM acetylated cytochrome *c* was measured in the absence and presence of 400 U ml^{-1} SOD and the difference was used to calculate the rate ($\varepsilon_{550-540\ nm}$: 19.1 mM^{-1} cm^{-1}) [36]. Kinetic data were usually recorded for 10 min at an interval of 13 readings min^{-1}.

2.4. High-resolution respirometry

The effect of various substances on the succinate-oxidase and NADH-oxidase was monitored at 37 °C using an Oxygraph-2k system (Oroboros, Innsbruck, Austria), equipped with two chambers and DatLab software version 4.2. We matched exactly the conditions (especially the molar ratio of SMP and applied inhibitors) to the corresponding ROS measurements. Therefore, SMP were applied at 240 μg protein to 2 ml buffer containing 75 mM sodium phosphate, 1 mM EDTA, and 1 mM $MgCl_2$ (pH 7.2). The succinate-oxidase was usually measured with 100 μM succinate, the NADH-oxidase with 250 μM NADH as substrates. The activity in the presence of the respective inhibitor concentrations was related to the uninhibited oxygen-consumption. Shown are mean values of duplicates.

3. Results

3.1. Mammalian complex II produces ROS at subsaturating succinate concentrations upon inhibition of the ubiquinone reductase activity

Recently, Quinlan and colleagues could show that mammalian complex II can be a major source of reactive oxygen species when the downstream respiratory chain is blocked [30]. In their investigation with isolated skeletal muscle mitochondria they observed that the ROS generation, measured as the release of hydrogen peroxide, was maximal at subsaturating succinate concentrations (peak at ~400 μM) and that it was almost completely suppressed by saturating concentration of the dicarboxylate. In a first set of experiments, we checked if complex II in uncoupled submitochondrial particles from bovine heart mitochondria produces ROS under corresponding conditions. To gain mechanistic insight into the ROS-production by complex II, uncoupled SMP as a model system have the advantages that (1) the determination of the ROS production is not attenuated by the antioxidative defence systems that are present in the matrix of mitochondria, (2) both H_2O_2 and superoxide production can be quantified, (3) the substrate concentration can be reliably adjusted (in intact mitochondria the matrix concentration of succinate is not identical to the one applied, since it is influenced by the activities of the dicarboxylate carrier and other TCA cycle enzymes) and (4) uncoupled SMP do not produce superoxide at complex I due to RET (this readily occurs in intact mitochondria especially at higher succinate concentrations and complicates the interpretation of the

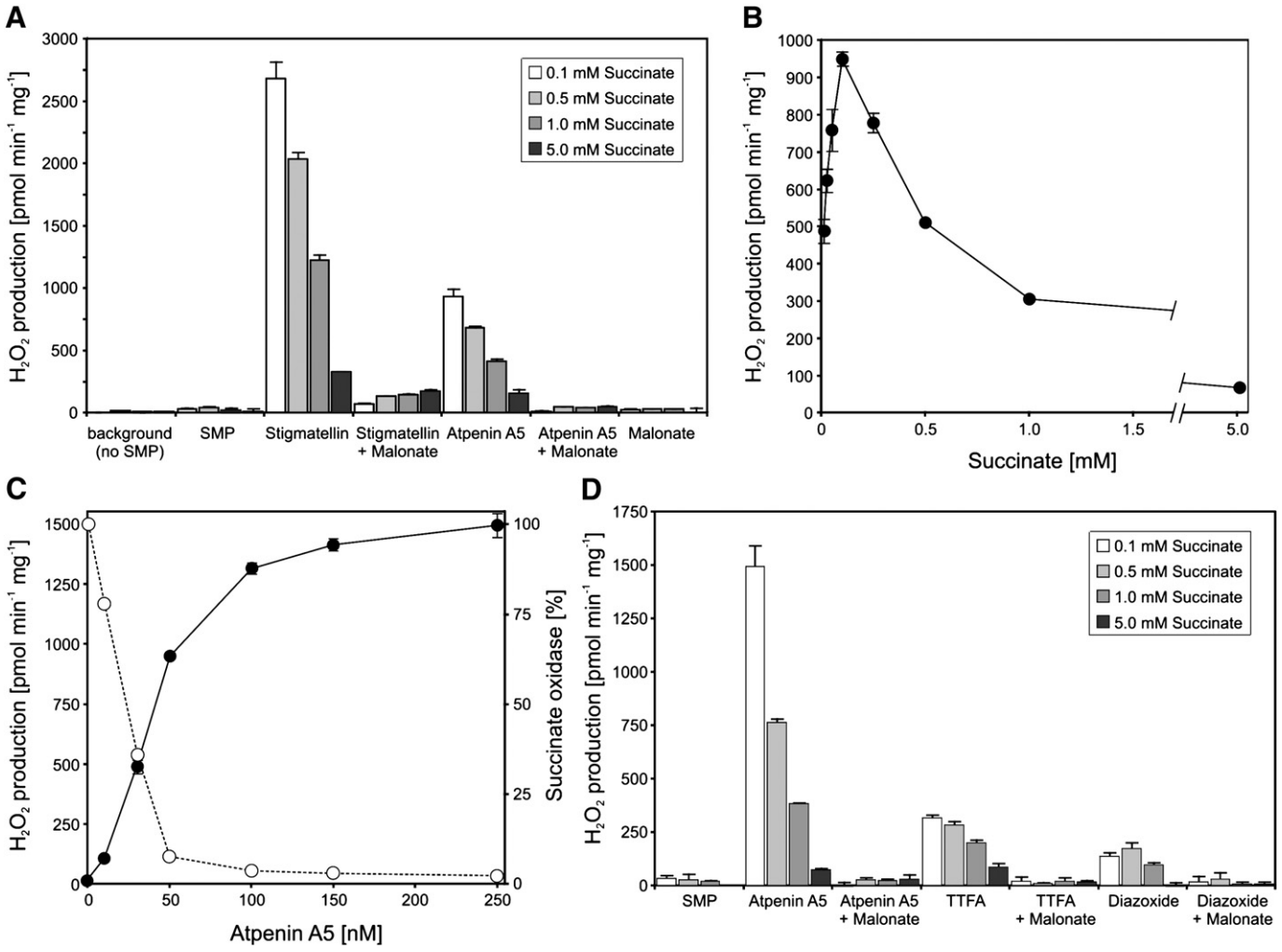

Fig. 1. Complex II produces ROS at subsaturating succinate concentrations upon inhibition of ubiquinone reductase activity. The total ROS production of SMP prepared from bovine heart mitochondria (0.12 mg protein ml^{-1}) was measured as H_2O_2 production in the presence of 400 U ml^{-1} SOD and using the Ampliflu Red™ assay as described in the Material and methods section. In general, mean values of three measurements and standard deviations (S.D.) are shown. (A) The succinate oxidase of SMP was fueled by different concentrations of succinate (as indicated) and the effect of the complex II Q-site inhibitor atpenin A5 (50 nM) and the complex III Q_o-site inhibitor stigmatellin (1 μM) on H_2O_2 production was determined. 1.5 mM malonate that binds competitively to the dicarboxylate site of complex II suppressed ROS production. (B) Effect of different succinate concentrations (0.01–5 mM) on the atpenin A5 induced ROS production. (C) The effect of different atpenin A5 concentrations on ROS production (filled circles, continuous line) and succinate oxidase activity (open circles, dashed line) were compared. In both experiments, SMP were fueled by 100 μM succinate. The succinate oxidase activity (100% were equal to an oxygen consumption of 78.9 nmol O_2 min^{-1} mg^{-1}) of SMP (0.12 mg protein ml^{-1}) was measured with an Oxygrap-2k as detailed in the Material and methods section. Mean values of duplicates are shown. (D) Induction of the succinate-supported ROS-production by different complex II Q-site inhibitors. Succinate was supplied in the range of 0.1 to 5 mM. Complex II inhibitors were present at the following concentrations: 250 nM atpenin A5 (inhibiting the succinate oxidase by 98%), 100 μM TTFA (inhibiting the succinate oxidase by 61%), 100 μM diazoxide (inhibiting the succinate oxidase by 38%) and 1.5 mM malonate (inhibiting succinate oxidase in the range of 100% in the presence of 100 μM succinate to 94.5% in the presence of 5 mM succinate).

data). The total ROS production (i.e. primary $O_2^{\bullet-}$ and H_2O_2 production) of SMP was determined as the H_2O_2 production in the presence of SOD with the Ampliflu Red™/HRP assay (Fig. 1A). The unspecific background rate in the absence of SMP could be neglected. In agreement with our previous investigations [13,14], uninhibited SMP produced H_2O_2 at a very low rate, also at subsaturating succinate concentrations. When the succinate oxidase of the SMP was inhibited by the addition of the complex III inhibitor stigmatellin [37] or the specific complex II Q-site inhibitor atpenin A5 [38], a largely increased H_2O_2 production could be observed at subsaturating succinate concentrations (Fig. 1A). Both inhibitors block the electron transfer from the terminal FeS-cluster onto ubiquinone, i.e. the ubiquinone reductase activity of complex II: atpenin A5 directly by competition with ubiquinone [38,39], the complex III inhibitor stigmatellin indirectly by preventing the reoxidation of ubiquinol to ubiquinone that is then no longer available for this reaction. The ROS production peaked at around 100 μM succinate and declined with increasing and decreasing succinate concentrations (Fig. 1A and B). This concentration is close to the K_m value (130 μM) of succinate that has been determined for purified complex II isolated from bovine heart mitochondria [40] indicating that the ROS production under these conditions is maximal around half saturation of the dicarboxylate site. The H_2O_2 production was completely suppressed by the competitive succinate analogue malonate. The contemporaneous presence of 1 μM DQA, a high affinity complex I inhibitor [41], had no affect on the ROS production (results not shown), excluding any contribution of complex I. The H_2O_2 production in the presence of 1 μM stigmatellin was about 2.5-times higher than the H_2O_2 production in the presence of 50 nM atpenin A5 (Fig. 1A). The Q_o-site of complex III could be excluded as a ROS source since binding of stigmatellin prevents the formation of superoxide at this site [15,42]. Our previous study had shown that 50 nM atpenin A5 was sufficient to completely inhibit the succinate-oxidase activity of SMP [14]. However, when we titrated the inhibitory activity of atpenin A5 with the present batch of SMP and 100 μM succinate (Fig. 1C), we observed that 50 nM inhibited

the succinate oxidase by only ~92%. Both the inhibition of the succinate oxidase and the atpenin A5 induced H_2O_2 production could be increased by higher inhibitor concentrations (Fig. 1C). At 250 nM atpenin A5, the complex II activity was inhibited by ~98% and the H_2O_2 production reached values around 1500 pmol min^{-1} mg^{-1}. This was still about 40% lower than the complex II dependent (i.e. malonate-sensitive) H_2O_2 production in the presence of the complex III inhibitor stigmatellin (Fig. 1A). Since the succinate-oxidase was completely inhibited by 1 μM stigmatellin (results not shown), we checked if this additional inhibition of the succinate-oxidase was responsible for the increased H_2O_2 production. However, when 250 nM atpenin A5 and 1 μM stigmatellin were both present, the detected H_2O_2 production was somewhat lower than the rate in the presence of 250 nM atpenin A5 alone (results not shown). This indicates that the highest rates of ROS production at complex II occurred when the electron flow was blocked at a site further downstream of the Q-binding site of complex II. Also other inhibitors of complex II that bind to the Q-site like TTFA and diazoxide induced ROS production at subsaturating succinate concentrations (Fig. 1D). The lower rates of H_2O_2 production corresponded well to the lower inhibitory potential of both substances (100 μM TTFA inhibited succinate oxidase by 61%, 100 μM diazoxide by 38%). Inhibition of more than 60% of the ubiquinone:reductase activity seems to be a prerequisite to produce a significant rate of ROS by complex II — at least when electrons are supplied by succinate in the 'forward mode'.

3.2. Complex II mainly produces hydrogen peroxide upon inhibition of the ubiquinone–reductase activity

Next, we investigated whether mitochondrial complex II produces mainly superoxide or hydrogen peroxide as primary ROS. The generation of membrane-impermeable superoxide could not be detected with intact mitochondria [30], since it is probably mainly released into the mitochondrial matrix. With SMP, we measured the superoxide production as the SOD-sensitive reduction of acetylated cytochrome *c* and the total ROS production with the Ampliflu Red™ assay in the presence of SOD (Fig. 2). The primary H_2O_2 production can be deduced by subtracting the superoxide production from the total ROS production. To allow direct comparison of both rates, they are expressed as electron equivalents transferred onto molecular oxygen, i.e. 1 e^- per $O_2^{\bullet-}$ and 2 e^- per H_2O_2, respectively. In the presence of 100 μM succinate, the inhibition of complex II by atpenin A5 or a further downstream block by stigmatellin induced mainly the production of H_2O_2 (Fig. 2). The rate of superoxide production at pH 7.2 corresponded to about 21–26% of the total ROS production. At pH 8.0 the overall rate of ROS generation as well as the fraction of superoxide (about 29–31%) increased slightly under the applied conditions. This can be explained by the higher activity of complex II at pH 8.0 [40] and the increased stability of the superoxide anion at basic pH [43]. The data indicate that complex II with inhibited ubiquinone reductase activity mainly produces H_2O_2 at physiological pH values.

3.3. Attenuation of the complex II-dependent ROS-production by competitive TCA cycle intermediates

Prior investigations indicate that ROS are only produced by the flavin when the dicarboxylate site is unoccupied [29,30]. Since other TCA metabolites also are known to bind competitively to the dicarboxylate site of complex II [44–46], we checked how these substances influence the ROS production. First we analyzed the effect of fumarate, the product of succinate oxidation that binds with comparable affinity to the dicarboxylate site [27,40]. Also fumarate attenuated the atpenin A5 (Fig. 3A) and stigmatellin (Fig. 3B) induced ROS generation in a concentration dependent manner. Considering the similar affinity of both carboxylates, fumarate was more effective than succinate. For example the H_2O_2 production in the presence of 0.1 mM succinate and 0.1 mM fumarate (total carboxylate concentration 0.2 mM) was lower than in the presence of 0.1 mM fumarate and 0.5 mM succinate (total carboxylate concentration 0.6 mM) or 0.5 mM succinate alone (Fig. 3). These results suggest that fumarate attenuated the ROS generation by two distinguishable effects: (1) by occupying the dicarboxylate binding site and (2) by changing the redox potential of the succinate/fumarate pair which in turn should affect the redox states of all electron-transferring cofactors of complex II. Hence, it can be concluded that the more reduced the cofactors the higher is the rate of ROS generation.

Also other TCA cycle intermediates, namely malate, citrate and especially oxaloacetate that are known to bind competitively to the dicarboxylate site attenuated the stigmatellin and atpenin A5 induced ROS production (Fig. 4). When we titrated the inhibitory effect of malate (Fig. 4B) and oxaloacetate (Fig. 4C) on the atpenin A5-induced ROS generation and compared it to the inhibition of the succinate-oxidase

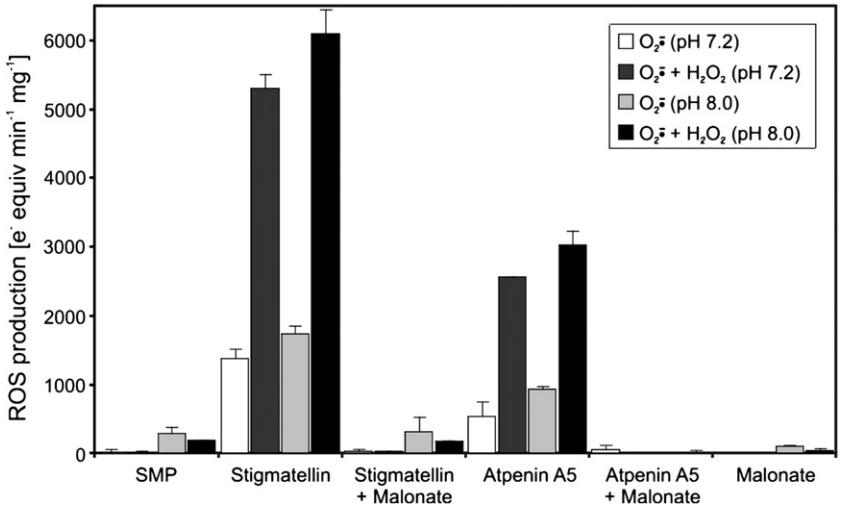

Fig. 2. Complex II with inhibited ubiquinone reductase produces mainly hydrogen peroxide. The superoxide production of SMP was determined as SOD-sensitive reduction of 25 μM acetylated cytochrome *c* and total ROS production as H_2O_2 production in the presence of 400 U ml^{-1} SOD as described in the Material and methods section. Both rates are expressed as the transfer of electron equivalents onto O_2, i.e. one e^- per superoxide and two e^- per H_2O_2. The ROS production was determined at pH 7.2 and 8.0. SMP were generally fueled by 100 μM succinate, inhibitors were added in the following concentrations: 1 μM stigmatellin, 250 nM atpenin A5 and 1.5 mM malonate. Mean values of three measurements and standard deviations (S.D.) are shown.

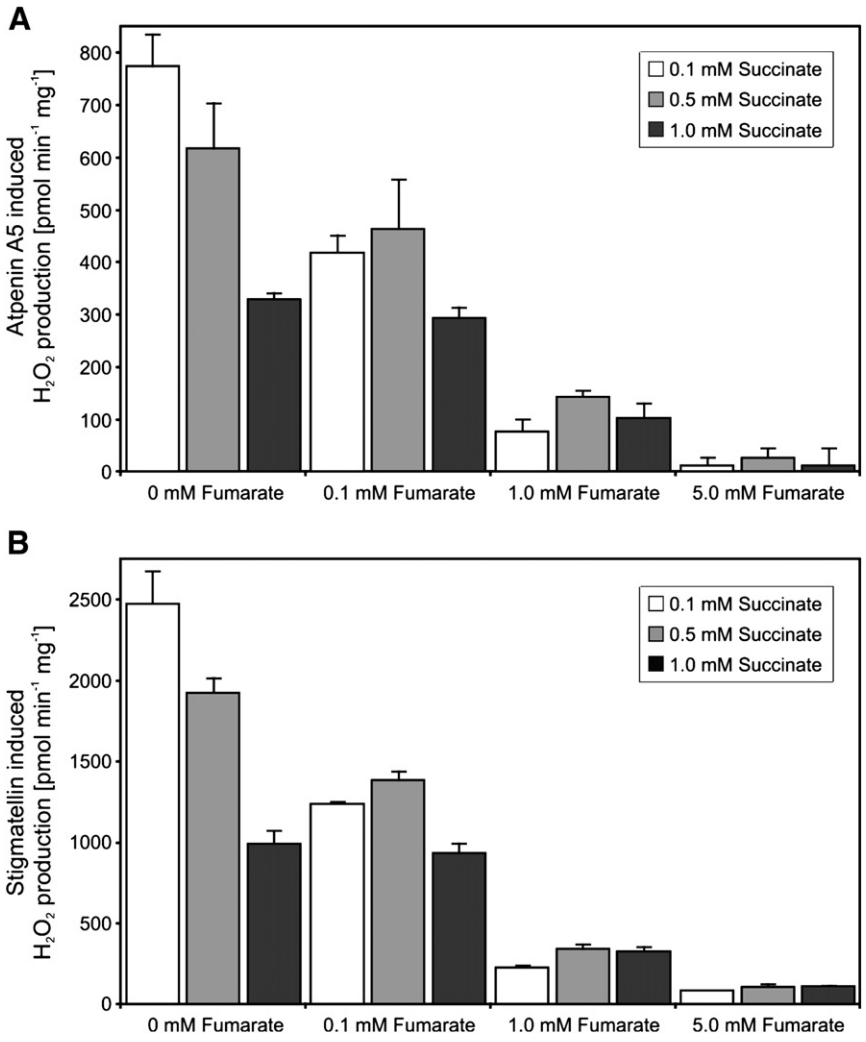

Fig. 3. Fumarate attenuates the ROS production of complex II. The determination of the H_2O_2 production and general conditions were the same as described in Fig. 1. (A) ROS production induced by 50 nM atpenin A5 was determined at different concentrations and ratios of the dicarboxylates succinate and fumarate. (B) General conditions as in (A), ROS production was induced by 1 μM stigmatellin. Mean values of three measurements and standard deviations (S.D.) are shown.

reflecting the complex II activity in SMP, we observed a remarkable congruence. Some discrepancy was only observed for malate concentrations above 1 mM. This could be attributed to the fact that on the one hand malate can be oxidized by complex II and therefore serves as a substrate of the complex II dependent oxidase activity of SMP [45–47]. On the other hand, the oxidation product of this reaction is the enol-form of oxaloacetate [47], a potent complex II inhibitor. This is also obvious from Fig. 4C that shows a half-maximal inhibition of the atpenin A5-induced ROS production and the succinate-oxidase by ~0.25 μM oxaloacetate.

3.4. Complex II is an independently inducible ROS generator

Finally, we checked, whether other respiratory chain complexes, especially complex I, the major electron supplier of the respiratory chain, affect the ROS generation by complex II. For this purpose, we analyzed the effect of various respiratory chain inhibitors on ROS generation when electrons were supplied exclusively by 250 μM NADH or 100 μM succinate or when both substrates were simultaneously present (Fig. 5). The DQA-induced ROS-generation at complex I was not influenced by the complex II inhibitors malonate and atpenin A5 that also had no inhibitory effect on the NADH-oxidase (results not shown). Conversely, the complex I inhibitor DQA had neither an effect on the atpenin A5 induced H_2O_2-generation in the presence of 100 μM succinate (Fig. 5) nor on the succinate-oxidase (results not shown). The concurrent presence of NADH somewhat reduced the atpenin A5 induced ROS-production, indicating that the redox-state of the Q-pool might have a minor effect under the applied conditions, i.e. that ubiquinol might be more effective than ubiquinone in displacing atpenin A5 in the Q-site. The maximal rate of H_2O_2 production measured in the presence of both substrates, 1 μM DQA and 250 nM atpenin A5 (13.7 nmol min^{-1} mg^{-1}) was quite similar to the sum of the individually induced rates (12.5 nmol min^{-1} mg^{-1}). These data indicate that complex II is an independently inducible ROS generator.

4. Discussion

Studying the molecular mechanisms of the mitochondrial ROS production is a timely topic [6], since it is becoming more and more evident that mitochondria-derived ROS are not only involved in the onset and progression of several diseases, but can also act as indispensable signaling molecules under non-pathological conditions [4,5]. It is also increasingly recognized that the sites of mitochondrial ROS production are inherently connected via a network of metabolic pathways and regulatory processes and that factors causing a down-regulation of ROS at one mitochondrial site might increase the ROS production at another site. A prime example is mammalian

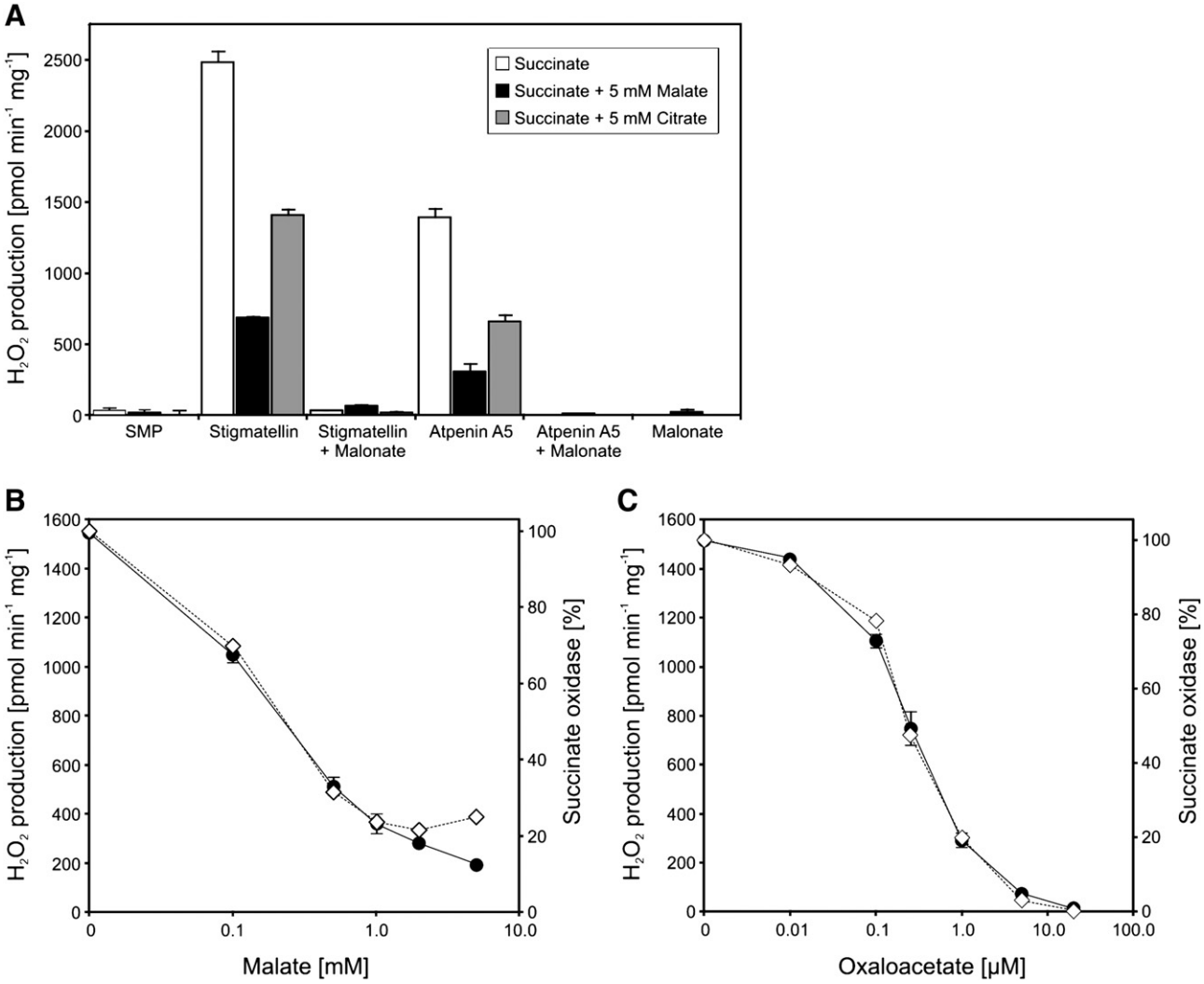

Fig. 4. Attenuation of the ROS production by different competitively binding TCA cycle intermediates. Determination of H_2O_2 production and general conditions were the same as in Fig. 1. Succinate was generally added at a concentration of 100 µM. (A) ROS production of complex II was induced by the addition of 1 µM stigmatellin or 250 nM atpenin A5. Dicarboxylates were added at 5 mM (malate and citrate), the inhibitor malonate at 1.5 mM, respectively. (B) Effect of different malate concentrations (0.1–5 mM) on the ROS production induced by 250 nM atpenin A5 (filled circles, continuous line) and the succinate oxidase (100% were equal to an oxygen consumption of 74.7 nmol O_2 min^{-1} mg^{-1}; open diamonds, dashed line). (C) Effect of different oxaloacetate concentrations (0.01–20 µM) on ROS production induced by 250 nM atpenin A5 (filled circles, continuous line) and succinate oxidase activity (100% were equal to an oxygen consumption of 72.7 nmol O_2 min^{-1} mg^{-1}; open diamonds, dashed line). Mean values of three measurements and standard deviations (S.D.) are shown for ROS production, mean values of duplicates are shown for succinate oxidase activity.

complex II [7] that can – under specific conditions – modulate the ROS production of complexes I and III in an ambivalent way [13,14] and can act – as recently shown by the investigation of Quinlan and colleagues [30] – as a significant source of ROS. This multifaceted interplay between complex II activity and ROS is also obvious from the role of complex II in the development of specific forms of cancer. On the one hand it is a plausible hypothesis that cancer associated complex II mutations increase the mitochondrial ROS production [19,22]. On the other hand complex II Q-site inhibitors like α-tocopheryl succinate [48] and mitochondrially targeted vitamin E succinate (MitoVES) [49,50] can be used to specifically induce cell death of cancer cells which involves the production of ROS [24,25]. To understand the role of complex II three key questions have to be answered: (1) What is/are the site(s) of ROS production, (2) which factors influence (i.e. promote or attenuate) the ROS production and (3) under which physiological and pathophysiological conditions does this occur.

Two sites have been proposed as the primary ROS source within mitochondrial complex II and related enzymes (SQRs and QFRs), the covalently bound FAD and the Q-site. The Q-site has been proposed based on studies in *Saccharomyces cerevisiae* [51,52] and a mixed contribution of FAD and the Q-site was suggested for complex II from the parasitic nematode *Ascaris suum* [53]. While a contribution of the Q-site seems reasonable for some Q-site mutants [54–57], the most likely source of ROS in functional complex II is the bound FAD [28–30,58]. This notion is supported by the trend of the succinate-dependent ROS generation [29,30] (Fig. 1B) and the differential effect of inhibitors that bind to the dicarboxylate and ubiquinone binding site, respectively (Fig. 6). It has to be noticed that *E. coli* SQR produces ROS in the absence of quinone-binding site inhibitors [29,30] while mammalian complex II only produces ROS in the forward mode (i.e. electrons are supplied by succinate) when its ubiquinone reductase activity is inhibited (Fig. 6A). Anyway, ROS production of both enzymes showed a similar dependence on the succinate concentration [29,30] (Fig. 1B). The trend of the succinate-dependent ROS production can be explained by a model that postulates two antithetic effects of succinate binding: on the one hand succinate binding is a prerequisite for the reduction of the redox-groups including the FAD that is presumably the primary source of ROS. This implies that increasing substrate concentrations cause an increasing ROS production (Fig. 6B). On the other hand, succinate binding hampers at the same time access of oxygen to the

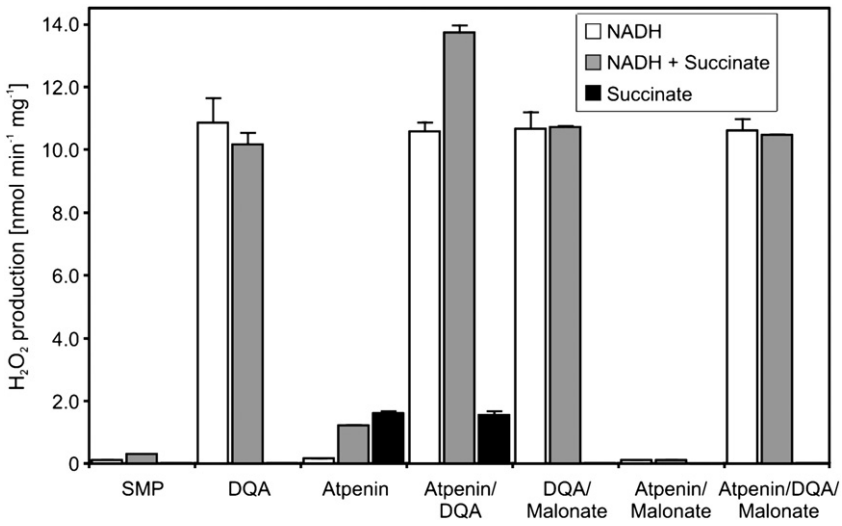

Fig. 5. Complex II is an independently inducible ROS generator. Determination of H_2O_2 production and general conditions were the same as in Fig. 1. The respiratory chain of SMP was either fueled by 250 μM NADH, 100 μM succinate or a combination of both substrates. Inhibitors were present at: 1 μM DQA, 250 nM atpenin A5 and 1.5 mM malonate. Mean values of three measurements and standard deviations (S.D.) are shown.

flavin with inverse concentration dependence, thereby limiting ROS production at saturating substrate concentrations. Since the kinetics of the succinate dehydrogenase reaction can be described by the Michaelis–Menten model, the trend of the succinate-dependent ROS-production can be simulated by superimposing two hyperbolic curves, one representing the succinate-dependent reduction of FAD and one inverted describing the succinate-dependent block of the oxygen access to the FAD (Fig. 6B). The resulting graph describes approximately the experimental results of the present and a previous study [30]: an increase at very low succinate concentrations, peaking at subsaturating succinate and a decrease at higher concentrations. Such a behavior would be hardly expected if the Q-site would be the primary ROS source, since in this case an increasing appearance of the ubiquinone species responsible for the ROS production, in this case most likely a semiquinone, should be promoted by increasing substrate concentrations. In addition, the present and a previous [30] study show that all

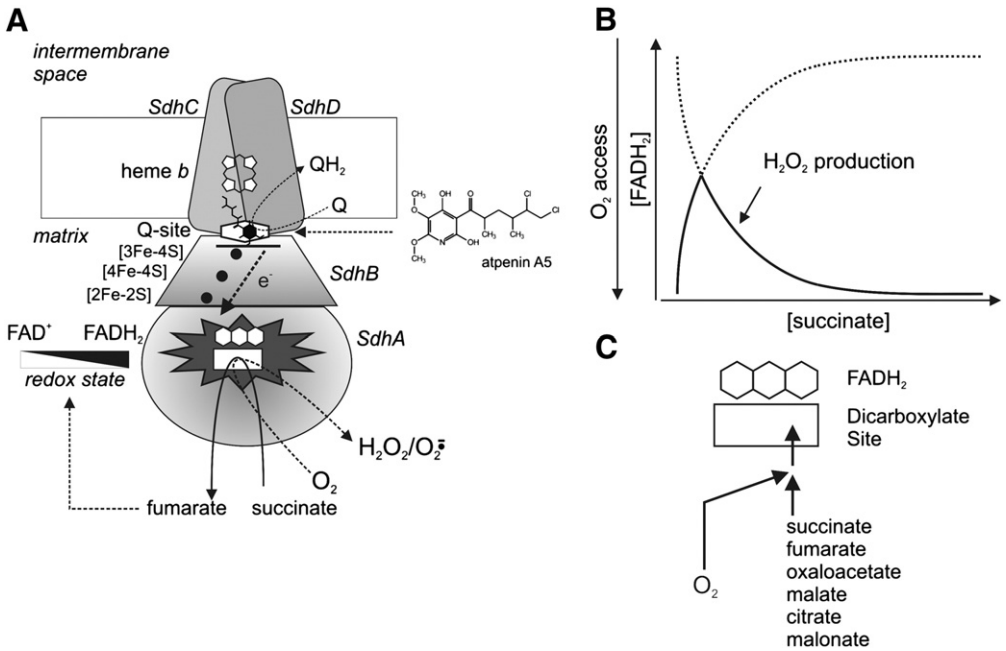

Fig. 6. Proposed model for ROS production of complex II. The model summarizes the findings of the present and some previous investigations (for details see text). (A) General structure of complex II and the site of ROS production. SdhA–D indicate the 4 subunits of complex II. The relative positions of the prosthetic groups and functional sites are indicated as black circles ([FeS]-clusters), a tricyclic structure (FAD), a stretched white hexagon (Q-site), a white rectangle (dicarboxylate binding site) and a schematic porphyrin ring (heme *b*). Upon binding of atpenin A5 to the Q-site, transfer of electrons is blocked and upstream redox-groups including the FAD become reduced. ROS (mainly hydrogen peroxide, but also some superoxide) can be only produced from $FADH_2$ if the dicarbocylate site is unoccupied. Also the binding of fumarate prevents the production of ROS. Fumarate additionally affects ROS generation by increasing the redox potential of the fumarate/succinate couple. (B) The trend of succinate-dependent ROS-production of complex II can be explained by the Michaelis–Menten type kinetic of the succinate–dehydrogenase reaction. Increasing succinate concentrations cause increased reduction of the FAD which is a prerequisite for ROS production. At the same time, the binding of succinate to the dicarboxylate site hampers access of oxygen with inverse concentration dependence. The two antithetic effects determine the ROS production (indicated by the solid line) at different succinate concentrations. (C) Besides succinate and fumarate, also other dicarboxylates that bind competitively to this substrate site can block the access of oxygen to the $FADH_2$. This supports that view that ROS are only produced if the dicarboxylate site is empty.

inhibitors and TCA metabolites that bind competitively to the dicarboxylate site of complex II attenuated ROS production while all inhibitors that bind to the Q-site of complex II increased ROS generation. At least a partial contribution of the Q-site or the [3Fe–4S] cluster might be conveyed from the fact that the ROS production in the presence of atpenin A5 was about 40% lower than in the presence of stigmatellin that blocks the respiratory chain further downstream at the Q_o-site of complex III. Such a reduction has been also observed by Quinlan et al. in intact skeletal rat mitochondria [30]. In the latter study it was shown that binding of atpenin A5 altered the catalytic properties of the dicarboxylate site causing a 25–45% decreased succinate dehydrogenase activity [30] which might account for the reduced ROS production. While our study strongly suggest that the FAD is a more likely site of ROS production – at least when the ubiquinone:reductase activity of complex II is inhibited – the data cannot exclude a partial contribution of the [3Fe4S] cluster or the Q-site.

Furthermore, the present study revealed that mitochondrial complex II with inhibited ubiquinone reductase activity mainly produces hydrogen peroxide as the primary reactive oxygen species. This is in contrast to *E. coli* SQR that produces almost exclusively superoxide [29]. The related *E. coli* QFR produced a mixture of superoxide and hydrogen peroxide that was shifted towards hydrogen peroxide at succinate concentrations above 1 mM [29]. Messner and Imlay concluded that QFR releases superoxide from the fully reduced flavin if the oxidized [2Fe–2S] cluster is available to sequester the semiquinone electron; otherwise this electron is rapidly transferred to the nascent superoxide and hydrogen peroxide is released. Yankovskaya et al. [58] suggested that the differential arrangement of redox potentials among the redox centers in *E. coli* SQR and QFR determines the rate and ROS preference at the flavin site. In SQR the high potential centers ([3Fe–4S] and heme *b*, which attract electrons and pull them away from the dicarboxylate site) are close to the Q-site, while in QFR, FAD and the [2Fe–2S] clusters have the highest redox potentials and hence accumulate electrons close to the dicarboxylate binding site. Having these considerations in mind, we found that the observed discrepancy between mammalian complex II and *E. coli* SQR can be related to the presence/absence of the Q-site inhibitor atpenin A5. In the presence of atpenin A5, the [2Fe–2S] cluster and the FAD should become much more reduced than in its absence which favors the formation of hydrogen peroxide over the release of superoxide. This would also imply that the fully reduced flavin is the primary source of ROS.

The general influence of the redox state of complex II cofactors on the rate of ROS production is also obvious from the measurements at different succinate/fumarate ratios. Both dicarboxylates bind with similar affinity to the dicarboxylate site of complex II [27,40], but fumarate has a more pronounced attenuating effect on ROS production. In the presence of atpenin A5 the redox state of the electron-transferring centers should be influenced by the preset potential of the succinate/fumarate couple and hence it can be deduced that the more reduced the redox centers are, the higher the rate of ROS production. The investigations of Messner and Imlay [29] and Yankovskaya et al. [58] implicate that also the redox states of the centers downstream of FAD modulate ROS production. In this regard, it might also matter whether the Q-site is occupied by an inhibitor or a ubiquinone molecule, which in turn might give an alternative explanation for the different maximal rates in the presence of atpenin A5 and stigmatellin.

An important finding of this and the previous investigation of Quinlan and colleagues [30] is that complex II can be a substantial source of ROS in the presence of 25–500 μM succinate. Concentrations in this range have been estimated for different tissues and mitochondria under normoxic conditions [59–62]. This means that any complex II Q-site inhibitor should in principle immediately induce the production of ROS from the flavin site. However, the ROS production is at least partly suppressed by other dicarboxylates that can bind competitively to the binding site of complex II (Fig. 6C). Especially oxaloacetate can effectively suppress ROS production by complex II. Inhibition of complex II also suppresses superoxide production at complex I by reverse electron transfer, indicating that oxaloacetate could be an important modulator of the mitochondrial ROS production under physiological conditions [12]. Finally, we showed that complex II is an independently inducible ROS generator that is not directly affecting the activity of the other respiratory chain complexes, although an indirect influence via the TCA cycle has to be considered. This might have important implications for the development of anticancer drugs targeted against the Q-site of complex II [24,25].

Acknowledgements

The authors thank Ulrich Brandt and Carla Jennewein for critically reading the manuscript and for helpful discussion. This work was supported by the DFG (Deutsche Forschungsgemeinschaft), SFB 815 *Redox-Regulation*, Project A02.

References

[1] M.P. Murphy, How mitochondria produce reactive oxygen species, Biochem. J. 417 (2009) 1–13.
[2] A.J. Kowaltowski, N.C. Souza-Pinto, R.F. Castilho, A.E. Vercesi, Mitochondria and reactive oxygen species, Free Radic. Biol. Med. 47 (2009) 333–343.
[3] M.D. Brand, The sites and topology of mitochondrial superoxide production, Exp. Gerontol. 45 (2010) 466–472.
[4] M.P. Murphy, A. Holmgren, N.G. Larsson, B. Halliwell, C.J. Chang, B. Kalyanaraman, S.G. Rhee, P.J. Thornalley, L. Partridge, D. Gems, T. Nystrom, V. Belousov, P.T. Schumacker, C.C. Winterbourn, Unraveling the biological roles of reactive oxygen species, Cell Metab. 13 (2011) 361–366.
[5] T. Finkel, Signal transduction by mitochondrial oxidants, J. Biol. Chem. 287 (2012) 4434–4440.
[6] S. Dröse, U. Brandt, Molecular mechanisms of superoxide production by the mitochondrial respiratory chain, Adv. Exp. Med. Biol. 748 (2012) 145–169.
[7] S. Dröse, Differential effects of complex II on mitochondrial ROS production and their relation to cardioprotective pre- and postconditioning, Biochim. Biophys. Acta 1827 (2013) 578–587.
[8] T.V. Votyakova, I.J. Reynolds, $\Delta\Psi_m$-Dependent and -independent production of reactive oxygen species by rat brain mitochondria, J. Neurochem. 79 (2001) 266–277.
[9] Y. Liu, G. Fiskum, D. Schubert, Generation of reactive oxygen species by the mitochondrial electron transport chain, J. Neurochem. 80 (2002) 780–787.
[10] A.J. Lambert, M.D. Brand, Superoxide production by NADH:ubiquinone oxidoreductase (complex I) depends on the pH gradient across the mitochondrial inner membrane, Biochem. J. 382 (2004) 511–517.
[11] F. Zoccarato, L. Cavallini, S. Bortolami, A. Alexandre, Succinate modulation of H_2O_2 release at NADH:ubiquinone oxidoreductase (complex I) in brain mitochondria, Biochem. J. 406 (2007) 125–129.
[12] F.L. Muller, Y.H. Liu, M.A. Abdul-Ghani, M.S. Lustgarten, A. Bhattacharya, Y.C. Jang, H. Van Remmen, High rates of superoxide production in skeletal–muscle mitochondria respiring on both complex I- and complex II-linked substrates, Biochem. J. 409 (2008) 491–499.
[13] S. Dröse, P.J. Hanley, U. Brandt, Ambivalent effects of diazoxide on mitochondrial ROS production at respiratory chain complexes I and III, Biochim. Biophys. Acta 1790 (2009) 558–565.
[14] S. Dröse, L. Bleier, U. Brandt, A common mechanism links differently acting complex II inhibitors to cardioprotection: modulation of mitochondrial reactive oxygen species production, Mol. Pharmacol. 79 (2011) 814–822.
[15] S. Dröse, U. Brandt, The mechanism of mitochondrial superoxide production by the cytochrome bc_1 complex, J. Biol. Chem. 283 (2008) 21649–21654.
[16] L. Bleier, S. Dröse, Superoxide generation by complex III: from mechanistic rationales to functional consequences, Biochim. Biophys. Acta (2013), http://dx.doi.org/10.1016/j.bbabio.2012.12.002.
[17] J. St Pierre, J.A. Buckingham, S.J. Roebuck, M.D. Brand, Topology of superoxide production from different sites in the mitochondrial electron transport chain, J. Biol. Chem. 277 (2002) 44784–44790.
[18] Q. Chen, E.J. Vazquez, S. Moghaddas, C.L. Hoppel, E.J. Lesnefsky, Production of reactive oxygen species by mitochondria: central role of complex III, J. Biol. Chem. 278 (2003) 36027–36031.
[19] T.M. Iverson, E. Maklashina, G. Cecchini, Structural basis for malfunction in complex II, J. Biol. Chem. 287 (2012) 35430–35438.
[20] T. Ishii, M. Miyazawa, H. Onouchi, K. Yasuda, P.S. Hartman, N. Ishii, Model animals for the study of oxidative stress from complex II, Biochim. Biophys. Acta 1827 (2013) 588–597.
[21] A.S. Hoekstra, J.P. Bayley, The role of complex II in disease, Biochim. Biophys. Acta 1827 (2013) 543–551.
[22] C. Bardella, P.J. Pollard, I. Tomlinson, SDH mutations in cancer, Biochim. Biophys. Acta 1807 (2011) 1432–1443.
[23] B.E. Baysal, Mitochondrial complex II and genomic imprinting in inheritance of paraganglioma tumors, Biochim. Biophys. Acta 1827 (2013) 573–577.
[24] S.J. Ralph, R. Moreno-Sanchez, J. Neuzil, S. Rodriguez-Enriquez, Inhibitors of succinate:quinone reductase/complex II regulate production of mitochondrial

reactive oxygen species and protect normal cells from ischemic damage but induce specific cancer cell death, Pharm. Res. 28 (2011) 2695–2730.
[25] K. Kluckova, A. Bezawork-Geleta, J. Rohlena, L. Dong, J. Neuzil, Mitochondrial complex II, a novel target for anti-cancer agents, Biochim. Biophys. Acta 1827 (2013) 552–564.
[26] G. Cecchini, Function and structure of complex II of the respiratory chain, Annu. Rev. Biochem. 72 (2003) 77–109.
[27] T.M. Iverson, Catalytic mechanisms of complex II enzymes: a structural perspective, Biochim. Biophys. Acta 1827 (2013) 648–657.
[28] J.A. Imlay, A metabolic enzyme that rapidly produces superoxide, fumarate reductase of *Escherichia coli*, J. Biol. Chem. 270 (1995) 19767–19777.
[29] K.R. Messner, J.A. Imlay, Mechanism of superoxide and hydrogen peroxide formation by fumarate reductase, succinate dehydrogenase, and aspartate oxidase, J. Biol. Chem. 277 (2002) 42563–42571.
[30] C.L. Quinlan, A.L. Orr, I.V. Perevoshchikova, J.R. Treberg, B.A. Ackrell, M.D. Brand, Mitochondrial complex II can generate reactive oxygen species at high rates in both the forward and reverse reactions, J. Biol. Chem. 287 (2012) 27255–27264.
[31] S. Dröse, U. Brandt, P.J. Hanley, K^+-independent actions of diazoxide question the role of inner membrane K_{ATP} channels in mitochondrial cytoprotective signaling, J. Biol. Chem. 281 (2006) 23733–23739.
[32] T.V. Votyakova, I.J. Reynolds, Detection of hydrogen peroxide with Amplex Red: interference by NADH and reduced glutathione auto-oxidation, Arch. Biochem. Biophys. 431 (2004) 138–144.
[33] S. Dröse, A. Galkin, U. Brandt, Measurement of superoxide formation by mitochondrial complex I of *Yarrowia lipolytica*, Methods Enzymol. 456 (2009) 475–490.
[34] M.J. Zhou, Z.J. Diwu, N. PanchukVoloshina, R.P. Haugland, A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases, Anal. Biochem. 253 (1997) 162–168.
[35] A. Azzi, C. Montecucco, C. Richter, The use of acetylated ferricytochrome *c* for the detection of superoxide radicals produced in biological membranes, Biochem. Biophys. Res. Commun. 65 (1975) 597–603.
[36] A. Stroh, O. Anderka, K. Pfeiffer, T. Yagi, M. Finel, B. Ludwig, H. Schägger, Assembly of respiratory complexes I, III, and IV into NADH oxidase supercomplex stabilizes complex I in *Paracoccus denitrificans*, J. Biol. Chem. 279 (2004) 5000–5007.
[37] G. von Jagow, T.A. Link, Use of specific inhibitors on the mitochondrial bc_1 complex, Methods Enzymol. 126 (1986) 253–271.
[38] H. Miyadera, K. Shiomi, H. Ui, Y. Yamaguchi, R. Masuma, H. Tomoda, H. Miyoshi, A. Osanai, K. Kita, S. Omura, Atpenins, potent and specific inhibitors of mitochondrial complex II (succinate–ubiquinone oxidoreductase), Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 473–477.
[39] R. Horsefield, V. Yankovskaya, G. Sexton, W. Whittingham, K. Shiomi, S. Omura, B. Byrne, G. Cecchini, S. Iwata, Structural and computational analysis of the quinone-binding site of complex II (succinate–ubiquinone oxidoreductase) — a mechanism of electron transfer and proton conduction during ubiquinone reduction, J. Biol. Chem. 281 (2006) 7309–7316.
[40] V.G. Grivennikova, E.V. Gavrikova, A.A. Timoshin, A.D. Vinogradov, Fumarate reductase activity of bovine heart succinate–ubiquinone reductase. New assay system and overall properties of the reaction, Biochim. Biophys. Acta 1140 (1993) 282–292.
[41] R.M. Hollingworth, K.I. Ahammadsahib, G. Gadelhak, J.L. McLaughlin, New inhibitors of complex I of the mitochondrial electron transport chain with activity as pesticides, Biochem. Soc. Trans. 22 (1994) 230–233.
[42] F.L. Muller, A.G. Roberts, M.K. Bowman, D.M. Kramer, Architecture of the Q_o site of the cytochrome bc_1 complex probed by superoxide production, Biochemistry 42 (2003) 6493–6499.
[43] B.H.J. Bielski, D.E. Cabelli, R.L. Arudi, A.B. Ross, Reactivity of HO_2/O_2^- radicals in aqueous solution, J. Phys. Chem. Ref. Data 14 (1985) 1041–1100.
[44] M. Gutman, E.B. Kearney, T.P. Singer, Control of succinate dehydrogenase in mitochondria, Biochemistry 10 (1971) 4763–4770.
[45] E.B. Kearney, B.A.C. Ackrell, M. Mayr, Tightly bound oxalacetate and activation of succinate dehydrogenase, Biochem. Biophys. Res. Commun. 49 (1972) 1115–1121.
[46] B.A.C. Ackrell, E.B. Kearney, M. Mayr, Studies on succinate–dehydrogenase. 24. Role of oxalacetate in regulation of mammalian succinate–dehydrogenase, J. Biol. Chem. 249 (1974) 2021–2027.
[47] Y.O. Belikova, A.B. Kotlyar, A.D. Vinogradov, Oxidation of malate by the mitochondrial succinate–ubiquinone reductase, Biochim. Biophys. Acta 936 (1988) 1–9.
[48] L.F. Dong, P. Low, J.C. Dyason, X.F. Wang, L. Prochazka, P.K. Witting, R. Freeman, E. Swettenham, K. Valis, J. Liu, R. Zobalova, J. Turanek, D.R. Spitz, F.E. Domann, I.E. Scheffler, S.J. Ralph, J. Neuzil, α-Tocopheryl succinate induces apoptosis by targeting ubiquinone-binding sites in mitochondrial respiratory complex II, Oncogene 27 (2008) 4324–4335.
[49] L.F. Dong, V.J.A. Jameson, D. Tilly, L. Prochazka, J. Rohlena, K. Valis, J. Truksa, R. Zobalova, E. Mandavian, K. Kluckova, M. Stantic, J. Stursa, R. Freeman, P.K. Witting, E. Norberg, J. Goodwin, B.A. Salvatore, J. Novotna, J. Turanek, M. Ledvina, P. Hozak, B. Zhivotovsky, M.J. Coster, S.J. Ralph, R.A.J. Smith, J. Neuzil, Mitochondrial targeting of α-tocopheryl succinate enhances its pro-apoptotic efficacy: a new paradigm for effective cancer therapy, Free Radic. Biol. Med. 50 (2011) 1546–1555.
[50] L.F. Dong, V.J.A. Jameson, D. Tilly, J. Cerny, E. Mahdavian, A. Marin-Hernandez, L. Hernandez-Esquivel, S. Rodriguez-Enriquez, J. Stursa, P.K. Witting, B. Stantic, J. Rohlena, J. Truksa, K. Kluckova, J.C. Dyason, M. Ledvina, B.A. Salvatore, R. Moreno-Sanchez, M.J. Coster, S.J. Ralph, R.A.J. Smith, J. Neuzil, Mitochondrial targeting of vitamin E succinate enhances its pro-apoptotic and anti-cancer activity via mitochondrial complex II, J. Biol. Chem. 286 (2011) 3717–3728.
[51] J. Guo, B.D. Lemire, The ubiquinone-binding site of the *Saccharomyces cerevisiae* succinate–ubiquinone oxidoreductase is a source of superoxide, J. Biol. Chem. 278 (2003) 47629–47635.
[52] S.S.W. Szeto, S.N. Reinke, B.D. Sykes, B.D. Lemire, Ubiquinone-binding site mutations in the *Saccharomyces cerevisiae* succinate dehydrogenase generate superoxide and lead to the accumulation of succinate, J. Biol. Chem. 282 (2007) 27518–27526.
[53] M.P. Paranagama, K. Sakamoto, H. Amino, M. Awano, H. Miyoshi, K. Kita, Contribution of the FAD and quinone binding sites to the production of reactive oxygen species from *Ascaris suum* mitochondrial complex II, Mitochondrion 10 (2010) 158–165.
[54] N. Senoo-Matsuda, K. Yasuda, M. Tsuda, T. Ohkubo, S. Yoshimura, H. Nakazawa, P.S. Hartman, N. Ishii, A defect in the cytochrome *b* large subunit in complex II causes both superoxide anion overproduction and abnormal energy metabolism in *Caenorhabditis elegans*, J. Biol. Chem. 276 (2001) 41553–41558.
[55] J.Z. Huang, B.D. Lemire, Mutations in the *C. elegans* succinate dehydrogenase iron–sulfur subunit promote superoxide generation and premature aging, J. Mol. Biol. 387 (2009) 559–569.
[56] T. Ishii, M. Miyazawa, A. Onodera, K. Yasuda, N. Kawabe, M. Kirinashizawa, S. Yoshimura, N. Maruyama, P.S. Hartman, N. Ishii, Mitochondrial reactive oxygen species generation by the SDHC V69E mutation causes low birth weight and neonatal growth retardation, Mitochondrion 11 (2011) 155–165.
[57] K.M. Owens, N. Aykin-Burns, D. Dayal, M.C. Coleman, F.E. Domann, D.R. Spitz, Genomic instability induced by mutant succinate dehydrogenase subunit D (SDHD) is mediated by $O_2^{-\bullet}$ and H_2O_2, Free Radic. Biol. Med. 52 (2012) 160–166.
[58] V. Yankovskaya, R. Horsefield, S. Tornroth, C. Luna-Chavez, C. Leger, B. Byrne, G. Cecchini, S. Iwata, Architecture of succinate dehydrogenase and reactive oxygen species generation, Science 299 (2003) 700–704.
[59] S. Hoyer, C. Krier, Ischemia and the aging brain — studies on glucose and energy-metabolism in rat cerebral-cortex, Neurobiol. Aging 7 (1986) 23–29.
[60] R.J. Wiesner, P. Rosen, M.K. Grieshaber, Pathways of succinate formation and their contribution to improvement of cardiac-function in the hypoxic rat-heart, Biochem. Med. Metab. Biol. 40 (1988) 19–34.
[61] K. Brockmann, A. Bjornstad, P. Dechent, C.G. Korenke, J. Smeitink, J.M.F. Trijbels, S. Athanassopoulos, R. Villagran, O.H. Skjeldal, E. Wilichowski, J. Frahm, F. Hanefeld, Succinate in dystrophic white matter: a proton magnetic resonance spectroscopy finding characteristic for complex II deficiency, Ann. Neurol. 52 (2002) 38–46.
[62] A.A. Starkov, The role of mitochondria in reactive oxygen species metabolism and signaling, Ann. N. Y. Acad. Sci. 1147 (2008) 37–52.