Rhodoquinone and Complex II of the Electron Transport Chain in Anaerobically Functioning Eukaryotes

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Jaap J. Van Hellemond, Maciej Klockiewicz§, Cor P. H. Gaasenbeek, Marleen H. Roos¶, and Aloysius G. M. Tielenst

From the Laboratory of Veterinary Biochemistry and the Department of Parasitology and Veterinary Tropical Medicine, Institute of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, 3508 TD Utrecht, The Netherlands and the Institute for Animal Science and Health (ID-DLO), 8200 AB Lelystad, The Netherlands.

Many anaerobically functioning eukaryotes have an anaerobic energy metabolism in which fumarate is reduced to succinate. This reduction of fumarate is the opposite reaction to the sucinate-ubiquinone oxidoreductase, complex II of the aerobic respiratory chain. Prokaryotes are known to contain two distinct enzyme complexes and distinct quinones, menaquinone and ubiquinone (Q), for the reduction of fumarate and the oxidation of succinate, respectively. Parasitic helminths are also known to contain two different quinones, Q and rhodoquinone (RQ). This report demonstrates that RQ was present in all examined eukaryotes that reduce fumarate during anoxia, not only in parasitic helminths, but also in freshwater snails, mussels, lugworms, and oysters. It was shown that the measured RQ/Q ratio correlated with the importance of fumarate reduction in vivo. This is the first demonstration of the role of RQ in eukaryotes, other than parasitic helminths. Furthermore, throughout the development of the liver fluke Fasciola hepatica, a strong correlation was found between the quinone composition and the type of metabolism: the amount of Q was correlated with the use of the aerobic respiratory chain, and the amount of RQ with the use of fumarate reduction. It can be concluded that RQ is an essential component for fumarate reduction in eukaryotes, in contrast to prokaryotes, which use menaquinone in this process. Analyses of enzyme kinetics, as well as the known differences in primary structures of prokaryotic and eukaryotic complexes that reduce fumarate, support the idea that fumarate-reducing eukaryotes possess an enzyme complex for the reduction of fumarate, structurally related to the succinate dehydrogenase-type complex II, but with the functional characteristics of the prokaryotic fumarate reductases.

Living with hypoxia or even anoxia is an everyday experience for many organisms. Not only many prokaryotes, but many eukaryotic organisms as well can function (temporarily) without oxygen. Parasitic helminths, freshwater snails, and some lower marine organisms are known to be able to survive anaerobic conditions by adaptation of their energy metabolism. In addition to simple fermentation in which glucose is degraded to ethanol or lactate, most of these facultative anaerobic eukaryotes contain another fermentation variant, malate dismutation (7). Malate dismutation is found in both strictly and facultatively anaerobically functioning prokaryotes as well as in some eukaryotes that are capable of functioning anaerobically, like parasitic helminths (1), freshwater snails (2), mussels (3), oysters (4), and lugworms and other marine invertebrates (5). Although several variations of malate dismutation with various end products occur, the use of the production of succinate as an electron sink is universal. The reduction of malate to succinate occurs in two reactions that reverse part of the Krebs cycle, and the reduction of fumarate is the essential NADH-consuming reaction to maintain redox balance. Therefore, the possibility arises that during anoxia, fumarate reduction occurs by reversal of the succinate dehydrogenase (SDH) already present in the aerobic respiratory chain or else is catalyzed by a fumarate reductase (FRD) specifically synthesized for this function (reviewed in Refs. 6–8). Bacteria contain two distinct enzyme complexes, succinate-ubiquinone oxidoreductase (complex II) and menaquinol-fumarate oxidoreductase (FRD), for oxidizing succinate and reducing fumarate, respectively (7), although each enzyme will catalyze both reactions in vitro. These electron-transferring enzyme complexes of fumarate-reducing eukaryotes have not been studied extensively, but it has been shown that Haemonchus contortus possesses two different genes for the B-subunit of complex II that are differentially expressed during the development of this parasite (9). This differential expression during development was later confirmed for another parasitic worm, Ascaris suum, in which the existence of two different stage-specific forms of complex II was also demonstrated (10). The enzyme complexes responsible for fumarate reduction and the quinones of succinate-producing eukaryotes other than parasitic helminths, however, have not yet been studied.

Parasitic helminths are known to contain two distinct quinones, ubiquinone (Q) and rhodoquinone (RQ), for transport of electrons between the enzyme complexes of the respiratory chain. Free-living aerobic stages of several helminths contain a higher ratio of Q to RQ than parasitic anaerobic stages (11–14). However, evidence for a quantitative correlation between the amount of RQ present and the importance of fumarate reduc-

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§ Present address: Dept. of Parasitology, Faculty of Veterinary Medicine, Agricultural University, Grochowska 272, 03-849 Warsaw, Poland.
¶ To whom correspondence should be addressed: Laboratory of Veterinary Biochemistry, P. O. Box 80176, 3508 TD Utrecht, The Netherlands. Tel.: 31-30-2535380; Fax: 31-30-2535492; E-mail: t.tielsen@biochem.dgk.ruu.nl.

The abbreviations used are: SDH, succinate dehydrogenase; FRD, fumarate reductase; Q, ubiquinone; Q0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; Q10, ubiquinone-35; Q9, ubiquinone-45; Q10, ubiquinone-50; RQ, rhodoquinone; RQ10, rhodoquinone-50; HPLC, high pressure liquid chromatography.
Menaquinone serves this function (15, 16). Therefore, RQ is an essential component for fumarate reduction in eukaryotes, in contrast to prokaryotes, where it is lacking. Furthermore, it is still unknown whether RQ occurs only in parasitic helminths or is also present in other eukaryotes that reduce fumarate. In addition, the quinone composition and the importance of fumarate reduction in vivo are indicated by closed arrows. End products are shown in boxes. AcCoA, acetyl-CoA; CITR, citrate; FUM, fumarate; MAL, malate; Methy-lmal-CoA, methylmalonyl-CoA; ME, malic enzyme; 2 G, 2-oxoglu-tarate; OXAC, oxaloacetate; PEP, phosphoenolpyruvate; PEPCK, phos- phoenolpyruvate carboxykinase; PK, pyruvate kinase; PROP, propionate; Prop-CoA, propionyl-CoA; PYR, pyruvate; SUCC, succinate; Succ-CoA, succinyl-CoA.

FIG. 1. Generalized pathways of aerobic and anaerobic carbo-hydrate degradation in eukaryotes. Aerobic degradation is indicated by open arrows, whereas malate dismutation and simple fermentation are indicated by closed arrows. End products are shown in boxes. AcCoA, acetyl-CoA; CITR, citrate; FUM, fumarate; MAL, malate; Methy-lmal-CoA, methylmalonyl-CoA; ME, malic enzyme; 2 G, 2-oxoglu-tarate; OXAC, oxaloacetate; PEP, phosphoenolpyruvate; PEPCK, phos- phoenolpyruvate carboxykinase; PK, pyruvate kinase; PROP, propionate; Prop-CoA, propionyl-CoA; PYR, pyruvate; SUCC, succinate; Succ-CoA, succinyl-CoA.

Mitochondrial fractions were prepared by differential centrifugation. Homogenates from which the cell debris was removed (10 min at 4°C and 600 × g) were centrifuged for 20 min at 4°C and 14,000 × g. The mitochondrial pellet was resuspended in homogenization medium and used for assays. Mitochondrial fractions were used in all experiments, except when the available amount of tissue was limited. The results obtained with homogenates and mitochondrial fractions of adult F. hepatica were the same, except for the quinone concentration/mg of protein, which was higher in mitochondrial fractions.

Enzyme Assays—To ensure that changes in fumarate reduction activities are not caused by changes in quinone composition, but really reflect changes in the enzyme complex itself, we used the direct assay of fumarate reductase activity with reduced benzyl viologen as electron donor described by Ackrell et al. (17), which has been used to distinguish SDH and FRD in prokaryotes. Succinate dehydrogenase activity was determined as described by Hagerhall et al. (18). The measured activity was corrected for nonspecific reduction of Q₀ by performing control assays with malonate instead of succinate. All enzyme assays were performed immediately after fractionation of freshly collected material. Protein was determined by a Lowry method as reported by Bensadoun and Weinstein (19) using bovine serum albumin as a standard.

Quinone Determination—Quinones were extracted from lyophilized homogenates or lyophilized mitochondrial preparations according to Zhu et al. (20). Lyophilized samples were crushed into powder before extraction with pentane. Four subsequent extractions were performed each time using five times as much pentane as the wet weight of the sample (w/v). During the third extraction, the sample was sonicated for 15 s on ice. Extracts were pooled, evaporated to dryness, and dissolved in ethanol, after which they were kept in the dark until HPLC analysis. Quinones were separated according to the HPLC method of Takamiya et al. (12) using a reversed-phase RP18 HPLC column (Lichog 250 mm, 5 μm, end-capped; 4 × 250 mm, Merck, Darmstadt, Germany). The quinones were eluted using a linear gradient from 15 to 21% diisopropyl ether in methanol (v/v) in 24 min. The eluted quinones were identified by comparing their retention times with Q₀, Q₉, and Q₁₀ standards and purified RQ₁₀ of F. hepatica. In addition, RQ₀ of F. hepatica was identified as RQ₀ by mass spectrometric analysis. RQ of the other examined species demonstrated four characteristics identical to RQ of F. hepatica: (i) the retention time on reversed-phase HPLC (R₁₀ = 0.165 (22)); (ii) the retention value on TLC (21); (iii) the oxidized and reduced (by potassium borohydride) absorption spectra (230–600 nm), which were recorded using a split-beam spectrophotometer (Aminco DW2A); and (iv) a purple color in concentrated ethanol solution instead of the yellow color of ubiquinones.

The quinones were spectrophotometrically quantified at 275 nm (R₂₅ Q₀ = 126.8 (23) and R₂₅ Q₁₀ = 165 (22)). Q₀ was used as an internal standard and was added to each homogenate or mitochondrial fraction (followed by gentle sonication, three times for 10 s) before the samples were lyophilized. Quinone amounts were corrected for the recovery of the Q₀ internal standard (between 55% and 90% for all samples).

RESULTS AND DISCUSSION

Quinone Composition—The electron transport chains of many bacteria employ menaquinone when fumarate is the final electron acceptor (15, 16, 23–25). In parasitic helminths, which also utilize fumarate as final electron acceptor during anaerobiosis, Allen (11) demonstrated, however, the presence of RQ. Since RQ is present mainly in aerobic, fumarate-reducing stages of parasitic helminths, it was suggested that rhodoquinol functions as electron donor in fumarate reduction, similar to menaquinol in fumarate reduction in other organisms (11). It is unknown whether RQ occurs only in parasitic helminths or is present in other fumarate-reducing eukaryotes as well, which would imply an important difference between prokaryotic and eukaryotic fumarate reduction (8).
Rhodoquinone and Complex II in Eukaryotes

Quinones were determined in mitochondrial fractions, except for H. contortus L3, F. hepatica metacercariae, and D. viviparus, where the quinones of a homogenate were analyzed (see “Experimental Procedures” for details). Results of independent experiments are shown with standard deviations (n = 3).

| Species                      | nmol/mg protein | % of total quinones |
|------------------------------|-----------------|---------------------|
| Rat heart                    | 5.0 ± 1.4       | 44 ± 3              |
| Bovine heart                 | 8.8 ± 2.0       | 24 ± 5              |
| M. edulis abductor muscle    | 0.06 ± 0.02     | 81 ± 4              |
| C. angulata abductor muscle  | 0.07 ± 0.01     | 17 ± 11             |
| A. marina body wall muscle   | 0.31 ± 0.02     | 83 ± 3              |
| L. stagnalis                 | 0.28 ± 0.08     | 1.4 —               |
| H. contortus L3              | 1.35 ± 0.17     | —                   |
| H. contortus adult           | 0.10 ± 0.01     | 0.01 13             |
| F. hepatica metacercariae    | 0.43 ± 0.05     | 0.02 24             |
| F. hepatica adult           | 0.03 ± 0.02     | 0.08 10             |
| D. viviparus                 | 0.58 ± 0.02     | 0.03 13             |

* Values represent percentage RQ of the total quinones (RQ plus Q).

All examined mitochondria from eukaryotes contain Krebs cycle activity and a respiratory chain. All these mitochondria contained Q (Table I), which is an essential component of the aerobic respiratory chain. The amounts of Q present in bovine and rat heart mitochondria (Table I) were in accordance with other reports (26–28). The observed amounts of total quinones in the examined parasitic helminths were comparable to the amounts of quinone in A. suum and Paragonimus westermani, two other parasitic helminths (12, 13). The amounts of Q in the abductor muscles of M. edulis and C. angulata were lower than those in parasitic helminths.

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livers obtained from a slaughterhouse had a higher RQ content than adult F. hepatica isolated from experimentally infected male Wistar rats (compare Table I and Fig. 2). The reason for this difference is still unknown, but it might be correlated with the increased egg production in mature liver flukes, which requires an enhanced energy metabolism.

Our results demonstrated that RQ is an indispensable component for efficient electron transport in the anaerobic electron transport chain of eukaryotic organisms. This implies an important difference compared with fumarate reduction in prokaryotes, which utilize menaquinone instead of RQ for fumarate reduction. Both menaquinone and RQ have similar standard redox potentials, −74 and −63 mV, respectively, in contrast to Q (E°Q = +100 mV). These standard redox potentials correlate with the function of RQ and menaquinone in fumarate reduction and with the function of ubiquinone in succinate oxidation (8). Interestingly, however, Q and RQ are both benzoquinones, whereas menaquinone is a naphthoquinone. Therefore, the complexes of eukaryotes or prokaryotes that function in vivo as an FRD interact with structurally distinct quinones, suggesting a difference in enzyme structure as well. This possible difference in structure between fumarate-reducing enzyme complexes of eukaryotes and those of prokaryotes was therefore further investigated by analysis of their kinetic properties and diode-like behavior (see below).

Kinetic Properties of Complex II—In prokaryotes, two distinct enzyme complexes are known to exist, one to oxidize succinate (SDH) and another one to reduce fumarate (FRD) (but it should be realized that in vitro, both enzyme complexes are able to catalyze the reaction in both directions). Earlier investigations on parasitic helminths (9, 10) suggested that distinct enzyme complexes, which are adapted to their in vivo function, exist in eukaryotes as well.

When measured, the activity ratio of succinate oxidation and fumarate reduction reflects the catalytic capacity of the total amount of enzymes present as one cannot distinguish between a reversible reaction in a single type of enzyme and the occurrence of two distinct enzyme complexes. The activity ratio is, however, still informative because a low ratio indicates the presence of a fumarate-reducing type of enzyme complex. Analysis of the activity ratios of succinate oxidation and fumarate reduction in mitochondrial fractions showed that complexes II from rat and bovine heart mitochondria were twice (1.9 and 2.2, respectively) as active in the direction of succinate oxidation as that of fumarate reduction. This corresponds with their in vivo function, as these strictly anaerobically functioning mitochondria contain Krebs cycle activity and therefore have to oxidize succinate. Mitochondria of the other species listed in Table I, which all contain RQ, demonstrated a lower SDH/FRD activity ratio (0.09–0.35). On the other hand, significant differences in activity ratios were not detected between different stages of F. hepatica and H. contortus, although in vivo, the free-living stages of these parasites oxidize succinate, whereas the parasitic stages reduce fumarate. Apparently, the observed change in the activity ratio that was observed between free-living and parasitic stages of A. suum (10) does not occur in F. hepatica and H. contortus.

The low SDH/FRD activity ratios, which we observed in all fumarate-reducing eukaryotes (parasitic helminths as well as the lower marine organisms), are mainly caused by a markedly increased fumarate reduction activity compared with rat and bovine heart mitochondria. This correlates with the high fumarate reduction activity of mitochondria from fumarate-reducing eukaryotes and, in this respect, shows that the fumarate-reducing enzyme complexes of these eukaryotes resemble the FRDs of prokaryotes.

Diode-like Properties of Complex II—Sucheta et al. (38) demonstrated that the energy metabolism of the developing liver fluke changes gradually from an aerobic energy metabolism, in which Krebs cycle activity occurs, to an anaerobic energy metabolism, in which malate dismutation occurs and fumarate is reduced. From these experiments, it was calculated how many electrons are transported by ubiquinone (UQ) from complexes I and II to complex III in the aerobic respiratory chain as well as how many electrons are transported by RQ from complex I to complex II in the anaerobic electron transport chain. Calculated use of Q and RQ during development of the liver fluke, respectively; □ and △, the detected amounts of Q and RQ, respectively, in liver flukes isolated from experimentally infected male Wistar rats at certain time points after infection. The means of three independent experiments are shown with standard deviations.
showed diode-like behavior (negative order). Adult *A. suum* SDH-type complex II possessing diode-like behavior. The opposite (Fig. 3), although both completely dependent on fumarate reductase activity (32, 40), showed diode-like behavior (negative order). Adult *H. contortus* (Fig. 3) and *D. viviparus*, on the other hand, which are not solely dependent on fumarate reductase activity (9, 41), showed positive-order kinetics and not the diode-like behavior. Hence, the absence or presence of the diode-like behavior in crude membrane preparations from these eukaryotic organisms gives no indication as to the importance of fumarate reduction in vivo.

**Comparison of Prokaryotic and Eukaryotic Enzyme Complexes That Reduce Fumarate—** Recent reports suggested that eukaryotes that reduce fumarate also contain, in addition to an enzyme complex for succinate oxidation, a distinct enzyme complex for fumarate reduction (9, 10). It can now be concluded that these enzyme complexes for fumarate reduction in eukaryotes differ significantly from the prokaryotic type of FRD since (i) all known primary structures of eukaryotic enzyme complexes functioning in vivo in the direction of fumarate reduction demonstrate a higher amino acid sequence similarity to mammalian and *E. coli* SDHs than to prokaryotic FRD (1, 9, 42, 43); and (ii) eukaryotic complexes that reduce fumarate interact with a benzoquinone (rho do quinone), whereas prokaryotic FRDs interact with a naphthoquinone (menaquinone). Therefore, in addition to the two known types of enzyme complexes in prokaryotes (SDH and FRD), which differ in primary structure, activity ratio of succinate oxidation and fumarate reduction, diode-like behavior, and interacting quinone, another type of complex exists. This type is present (next to the SDH-type complex) in fumarate-reducing eukaryotes and belongs to the FRD category with respect to the functional properties of the complex, like the SDH/FRD activity ratio and the relatively low standard redox potential of the interacting quinone, *RQ* (*E*°′ = −63 mV). On the other hand, this type of enzyme belongs to the SDH category of complex II with respect to its primary structure and the type of interacting quinone, a benzoquinone, as our results demonstrated that in eukaryotes the essential component in fumarate reduction is *RQ*.

Our results support the idea that fumarate-reducing eukaryotes possess an enzyme complex for the reduction of fumarate, which is structurally related to SDH-type complex II, but has the functional characteristics of prokaryotic FRDs. For definitive conclusions, however, on the differences between enzyme complexes for succinate oxidation and fumarate reduction in eukaryotes, further experiments, like sequence comparisons and analysis of the kinetic properties of the purified enzymes, will be necessary.

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