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STUDIES ON THE ROLE OF UBIQUINONE IN THE CONTROL OF THE MITOCHONDRIAL RESPIRATORY CHAIN

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This study examines the possible role of Coenzyme Q (CoQ, ubiquinone) in the control of mitochondrial electron transfer. The CoQ concentration in mitochondria from different tissues was investigated by HPLC. By analyzing the rates of electron transfer as a function of total CoQ concentration, it was calculated that, at physiological CoQ concentration NADH cytochrome c reductase activity is not saturated. Values for theoretical V_{max} could not be reached experimentally for NADH oxidation, because of the limited miscibility of CoQ_{10} with the phospholipids. On the other hand, it was found that CoQ_3 could stimulate α-glycerophosphate cytochrome c reductase over three-fold. Electron transfer being a diffusion-coupled process, we have investigated the possibility of its being subjected to diffusion control. A reconstruction study of Complex I and Complex III in liposomes showed that NADH cytochrome c reductase was not affected by changing the average distance between complexes by varying the protein: lipid ratios. The results of a broad investigation on ubiquinol cytochrome c reductase in bovine heart submitochondrial particles indicated that the enzymic rate is not diffusion-controlled by ubiquinol, whereas the interaction of cytochrome c with the enzyme is clearly diffusion-limited.

KEY WORDS: Ubiquinone, mitochondria, diffusion, ubiquinol cytochrome c reductase, kinetics.

INTRODUCTION

Ubiquinone (Coenzyme Q, CoQ) is an essential component of the mitochondrial respiratory chain that functions as a mobile electron transfer component between membranous flavoprotein dehydrogenases and the cytochrome bc1 complex in the inner mitochondrial membrane.[1] Evidence supporting the random collisional nature of electron transfer has been collected by several approaches. Kinetic analysis of the rate of electron input to ubiquinone (V_r) and of electron output from reduced ubiquinone (V_o) established that it distributes electrons randomly from the dehydrogenase to the bc1 complex, behaving as a freely diffusable intermediate.[2] In fact the observed electron transfer rate (V^{obs}) follows a hyperbolic relation:

$$V_{obs} = V_o \cdot V_r / V_o + V_r$$

Although the ubiquinone concentration is in excess over that of the other electron transfer components in the respiratory chain, its substrate-like nature for the enzymes receiving electrons from and feeding electrons to the CoQ pool allows the legitimate question whether its concentration is saturating for maximal electron transfer activity.

Indications that this may not be the case are the following: (a) dilution of the mitochondrial inner membrane with excess phospholipids, so that the CoQ concentration in the membrane is lowered, proportionally reduces electron transfer turnover from NADH or succinate to cytochrome *c*;[3] (b) under pathological conditions, a decreased CoQ content in mitochondria was found associated with decreased electron transfer activity;[4] (c) the CoQ levels in mitochondria, as well as in other organelles, may be changed under several dietary conditions.[5]

In view of its one-electron carrier characteristics with ubisemiquinone as an obligatory intermediate in most quinone-mediated reactions,[6] Coenzyme Q is believed to function as an antioxidant[7] as well as a prooxidant;[8] in analogy with other antioxidants, as vitamin E, it has been questioned whether ubiquinone content may decrease under conditions of oxidation stress, due to formation of oxidation products.[9]

In such case, if CoQ concentration were not saturating, the obvious consequence would be a decrease of electron transfer through the respiratory chain.

In this report we analyze theoretically as well as experimentally whether ubiquinone concentration is saturating for electron transfer in mitochondria. The results suggest that CoQ concentration is near its average affinity for its partner enzymes, but kinetic saturation of maximal electron transfer cannot be experimentally obtained due to the limited miscibility of functional (monomeric) ubiquinone in the membrane. On the other hand, the rate of lateral diffusion of CoQ in the membrane phospholipids does not appear to represent a limiting factor for the rate of electron transfer.

MATERIALS AND METHODS

Bovine heart mitochondria (BHM) and submitochondrial particles (SMP) were prepared as described elsewhere.[10] Other types of mitochondria were isolated according to the following references: liver mitochondria,[11] except fish liver mitochondria;[12] heart mitochondria;[13] brown adipose tissue mitochondria,[14] and brain mitochondria.[15]

Phospholipid vesicles were obtained[16] by sonication of soybean phospholipids (Asolectin from Associated Concentrates, New York) and purified according to.[17] The bc_1 complex from beef heart was isolated and purified as described elsewhere.[10]

Proteoliposomes containing Complexes I and III were prepared by cholate dialysis of a partially purified fraction from BHM, fraction R4B[18] with Asolectin.[19] The content of each complex in fraction R4B was calculated on the basis of its FMN and cytochrome c_1 content.

Phospholipid-enriched mi45tochondrial membranes were prepared by freezing and thawing a mitochondrial suspension together with Asolectin liposomes.[20] The mixture was loaded on a discontinuous sucrose gradient and centrifuged at 70,000 g for 14–16 hours at 4°C; the bulk of the phospholipids remained at the top of the gradient whereas the membranes were separated into distinct fractions depending on the phospholipid content.

Ubiquinol cytochrome *c* reductase, NADH cytochrome *c* reductase, alpha-glycerophosphate cytochrome *c* reductase and cytochrome *c* oxidase were assayed as described previously[10] for the former enzyme, using a Sigma Biochem dual wavelength spectrophotometer equipped with a rapid mixing device, following the absorbance changes of cytochrome c at 550 minus 540 nm (extinction coefficient 18 mM^{-1} cm^{-1}). NADH oxidase and ubiquinol oxidase were followed polarographically with a Clark oxygen electrode.

The ubiquinone concentration was determined after extraction from mitochondria[21] by HPLC according to ref.[22]

The FMN content was measured as acid-extractable flavin.[23] The cytochrome content was determined from the dithionite-reduced minus ferricyanide -oxidized spectrum in a Perkin-Elmer 559 spectrophotometer.[24]

Freeze-fracture electron microscopy was kindly performed by Prof. G. Biagini and Dr. F. Marinelli of the University of Ancona, using a Philips 301 electron microscope at 80 kV.

RESULTS AND DISCUSSION

Is CoQ concentration saturating for electron transfer?

Under nonsaturating conditions, the rate of electron transfer across the CoQ pool (V^{obs}) is a function of total ubiquinone concentration (Q_t) and of the maximal velocities and K_m for ubiquinone (ubiquinol) of ubiquinone reductases (V_{mr}, K_{mr}) and ubiquinol oxidase (V_{mo}, K_{mo}) according to the following equation (25):

$$V_{obs} = \frac{[(V_{mr} \cdot V_{mo})/(V_{mr} + V_{mo})] \cdot Q_t}{\{[(V_{mr} \cdot K_{mo}) + (V_{mo} \cdot K_{mr})]/(V_{mr} + V_{mo})\} + Q_t}$$

V_{obs} is hyperbolically related to Q_t and maximal turnovers of electron transfer are attained only at Q_t saturating both V_r and V_o.

In bovine heart mitochondria, the average values for V_{mr}, V_{mo}, K_{mr}, K_{mo}, are reported in Table I. A computed double reciprocal plot of V_{obs} vs. Q_t using the values of Table I is shown in Figure 1; the plot extrapolates to $V_{obs(max)}$ of 28.5 nmol/s.mg and to a "K_m" for CoQ_{10} of NADH-CoQ reductase of 7.5 mM in the phospholipids.

At physiological CoQ concentration ($\sim$ 8 mM in the phospholipids for BHM[26]), the computed NADH cytochrome *c* reductase activity is 14.7 nmol/s.mg, corresponding to 0.88 μmol/min.mg, which is close to the values obtained experimentally in mitochondrial membranes, and corresponding to 52% of $V_{obs(max)}$.

The CoQ content of different types of mitochondria, related to other mitochondrial components and enzyme activities, is widely different (Table 2): in the table the values are also compared with cytochrome *b* content and ubiquinol cytochrome *c* reductase activity. Studies are under way whether the different values of ubiquinone content correspond to different levels of saturation of electron transfer.

We have tested this theoretical behavior by an experimental approach. The α-glycerophosphate cytochrome *c* reductase of hamster brown adipose tissue mitochondria

TABLE I

Average values of the kinetic constants of ubiquinone reductases and ubiquinol oxidase in mitochondria and calculated values for CoQ pool function.

Activity	V_{max} (nmol/s.mg)	V_{obs} (nmol/s.mg) (at normal Q_t)	K_m (Q_{10}) (mM in lipids)	Reference
NADH -CoQ reductase	45		10	37
Succinate CoQ reductase	17		5	37, 38
QH_2 cyt. *c* reductase	78		3.3	39
NADH cyt. *c* reductase	28.5	14.7 (51.5%)	7.5	calculated
Succinate cyt. *c* reductase	13.8	9.0 (65%)	4.1	calculated

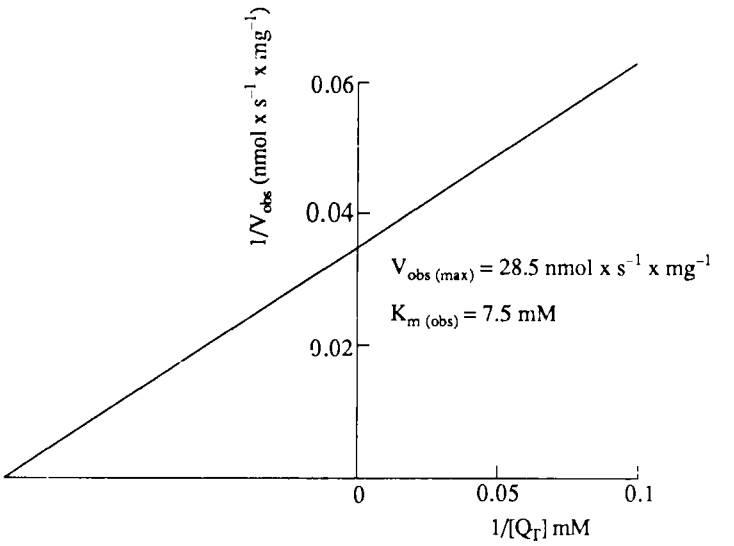

FIGURE 1 Computed double reciprocal plot of NADH cytochrome *c* reductase versus total ubiquinone concentration (Q_1) in bovine heart mitochondria using the values of Table 1.

was titrated with different levels of CoQ_3 (Figure 2). The strong stimulation by the quinone clearly indicates that the low levels of CoQ_9 and CoQ_{10} of these mitochondria are not saturating for maximal activity. Considering the basal activity as the expression of the levels of endogenous CoQ (0.75 nmol/mg protein, Table 2), an apparent K_m for CoQ is obtained of > 100 mM in the phospholipids; this very high value is

TABLE 2
Coenzyme Q and cytochrome *b* contents and ubiquinol cytochrome *c* reductase activity of different mitochondria.

Mitochondria	Coenzyme Q Type	Coenzyme Q Content (nmol per mg protein)	Cytochrome b (nmol per mg protein)	Ubiquinol cyt. c reductase activity (30°) (μmol/min.mg protein)
Hamster BAT*	Q9	0.54	0.27	5.7
	Q10	0.21		
Rat heart	Q9	2.95	0.28	1.5
	Q10	0.24		
Rat liver	Q9	1.32	0.13	2.0
	Q7,Q8,Q10	1.02		
Rat brain cortex	Q9	0.90	0.12	
	Q10	0.45		
Chicken heart	Q10	2.71	0.32	3.1
Chicken liver	Q10	2.04	0.19	2.7
Trout liver	Q10	0.81	0.085	1.2
Beef heart	Q10	1.83		
		4.0**	0.50**	3.1

*Brown adipose tissue
**From ref.[34]

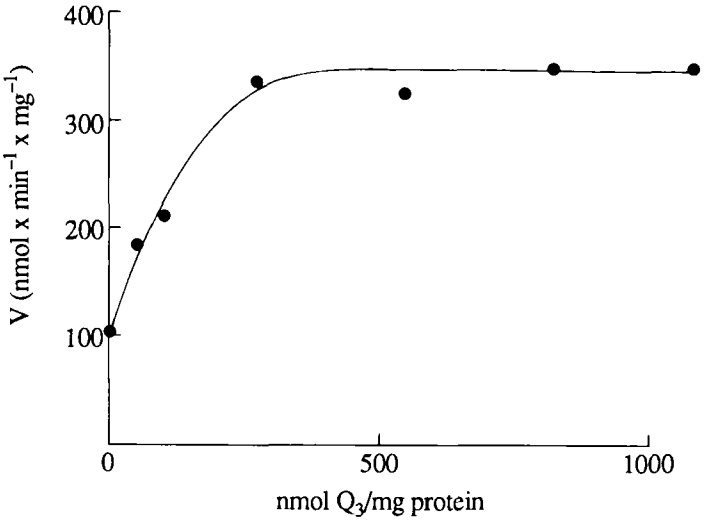

FIGURE 2 Effect of added CoQ_3 on α-glycerophosphate cytochrome *c* redutase of brown adipose tissue mitochondria of the hamster.

probably the result of incomplete solubilization of CoQ_3 in the mitochondrial membrane.[27]

This type of experimental approach is prevented for NADH cytochrome *c* reductase because this activity is inhibited by short chain ubiquinones.[28] For this reason we have used phospholipid enriched mitochondrial membranes[3] in order to incorporate long chain ubiquinones into the mitochondria.

We have increased the phospholipid content of rat liver mitochondria varying the ubiquinone content by fusing mitochondria with liposomes containing varying levels of ubiquinone-10. The fall of NADH cytochrome *c* reductase and NADH oxidase activities observed adding only phospholipids was avoided by incorporating phospholipids plus ubiquinone (Table 3). It is of interest that enrichment with ubiquinone levels higher than the physiological CoQ concentration is able to enhance respiratory activity above the control level.

The increase observed at 20 mM CoQ_{10} assuming a K_m in rat liver mitochondria as in bovine heart mitochondria, and considering a CoQ concentration of 7.5 mM in the phospholipids (Table 2) was from 50% to 69% of theoretical V_{max}, against a theoretical increase to 73%. The good correspondence of the values suggests that the theoretical elaboration was correct.

On the other hand, the experiment clearly points out that the values close to to

TABLE 3
NADH cytochrome *c* reductase activity of rat liver mitochondria fused with liposomes

Mitochondria	nmol/min.mg protein	% of control
Control	360	100
Phospholipid-enriched	201	56
Phospholipids + Q_{10}	482	135

TABLE 4
Survey of studies on CoQ localization in artificial systems and membranes

Technique	Results	Reference
Thermodynamic (partition)	Hydrophobic phase	40
Spectrophotometry (λ_{max})	Hydrophobic phase	41
Fluorescence quenching	Indeterminate in whole bilayer	27, 30
Diffusion coefficients	Low viscosity medium (mid-plane)	27, 42
EPR spin labels	Membrane midplane	43
Differential scanning calorimetry	Separate phase	44
NMR	Conflicting results	45–48
Chemical accessibility	Little availability at surface	47
	Availability to surface	49
Neutron diffraction	Separate phase	48
Linear dichroism	Oscillating between parallel and perpendicular to surface	29

kinetic V_{max} in NADH oxidation cannot be reached experimentally, because the miscibility of long chain ubiquinones with phospholipid bilayers is limited.

Data from the literature indicate that at high CoQ/phospholipid ratios the quinone forms separate phases, whose location with respect to the membrane is unclear (Table 4). Monomeric CoQ in the membrane, as appears for quinone sonicated with phospholipids at less than 20 mM (1.6 CoQ to phospholipids molar ratio), is probably located in the inner hydrophobic core of the bilayer.

Recent studies[29] by linear dichroism of ubiquinones in lipid micelles oriented in a magnetic field show a mixed orientation of ubiquinone molecules with the ring perpendicular to the membrane plane, but with two positions of the axis passing across the carbonyls, perpendicular to one another.

The most appropriate location of the ubiquinone molecule in the membrane is one in which the hydrophobic sidechain is located in the midplane, whilst the quinone ring oscillates from the midplane towards the surfaces (Figure 3). The availability of the quinone ring to quenching of membrane fluorophores located at different depths in the bilayer[27,30] agrees with the above interpretation.

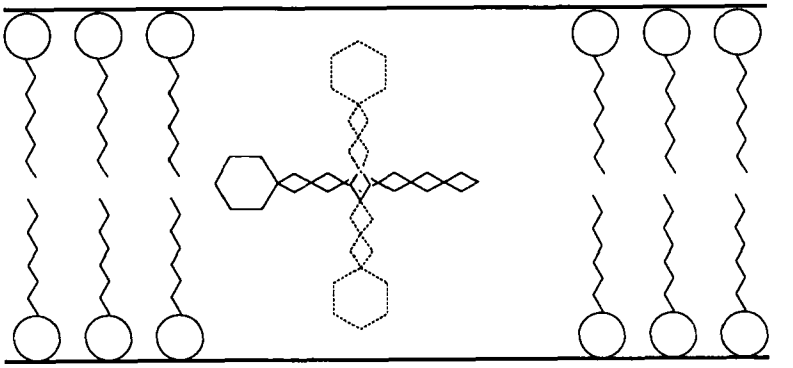

FIGURE 3 A model of ubiquinone localization in the phospholipid bilayer.

Is CoQ lateral diffusion rate-limiting for electron transfer?

Being electron transfer a diffusion coupled process,[31] the question may be related whether the rate of diffusion of the quinone in the pool between its partner enzymes is limiting for electron transfer. The random collision model of Hackenbrock and his coworkers[31] explicitly postulates that electron transfer is diffusion limited by ubiquinone.

The lateral diffusion coefficients of ubiquinone measured by fluorescence photobleaching recovery in mitochondrial membranes are *ca.* 10^{-9} cm^2/s[32] whereas those measured by fluorescence collisional quenching in lipid vesicles or mitochondrial membranes are in the range of 10^{-7} cm^2/s.[27,33]

The discrepancy between these diffusion coefficients is wide, and would be important to be clarified, because calculations of the time to cover the distance between respiratory enzymes would suggest that the rate of electron transfer is near the diffusion limit only in the first case.[34] We have therefore approached the problem by a direct kinetic approach.

In the first set of experiments we have titrated the NADH cytochrome *c* reductase activity of a reconstructed fraction containing complexes I and III (fraction R4B) at different phospholipid to protein ratios.

The crude NADH cytochrome *c* reductase contained 0.36 nmol FMN and 0.5 nmol cytochrome c_1 per mg protein. We have incorporated the fraction in liposomes by cholate dialysis at different phospholipid to protein ratios and with different levels of CoQ_6. The basic assumption of a random distribution of the complexes in the liposomes, so that the increased lipid protein ratios also correspond to an increased

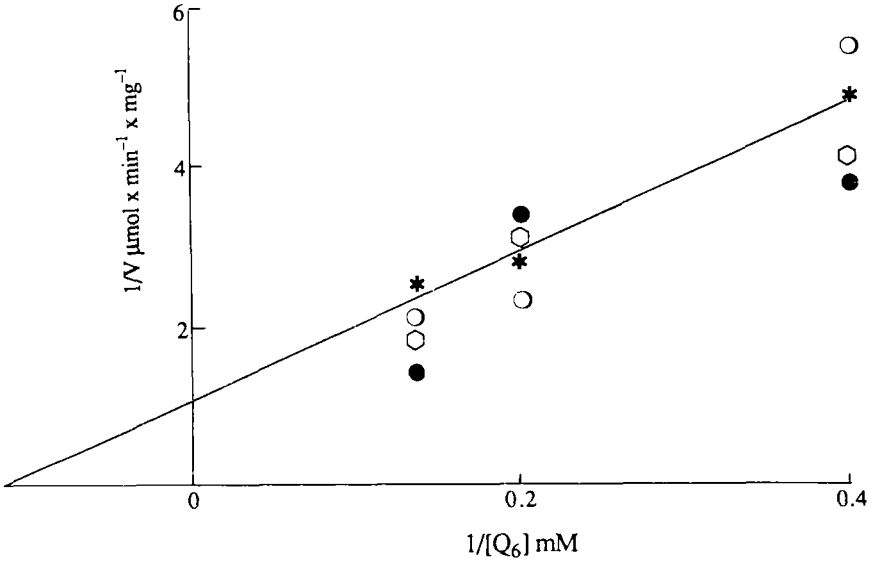

FIGURE 4 Effect of increasing the distance between Complexes I and III on NADH cytochrome c reductase at different ubiquinone-6 contents. The phospholipid to protein ratios were: (○), 10; (●), 20; (⬡), 40; (★), 80, corresponding to 30, 54, 76, 108 nm average distance between the complexes. Data are plotted as double reciprocal plots with respect to CoQ_6 concentrations.

average distance between complexes, was verified by freeze-fracture electron microscopy, showing a random distribution of the intramembrane particles.

The results in Figure 4 show that the turnovers of NADH cytochrome *c* reductase at each ubiquinone level are not affected by the distance between the complexes, at least within the experimental limit of 108 nm, if we except the scatter due to the fact that each experimental point corresponds to a different cholate dialysis experiment.

The experiment reveals adherence to pool behavior, because NADH cytochrome *c* reductase activity depends on CoQ concentration in the membrane. The V_{max} extrapolated at infinite Q_6 of 1 μmol/min. mg corresponds to a turnover based on FMN content of 46.3 s^{-1}, with a turnover time of 22 ms.

The time t for a particle to diffuse to a small target of diameter d over a distance l in two dimensions is given by:[35]

$$t = (l^2/2D) \cdot (\ln l/d - 3/4)$$

By applying this equation for $l = 108$ nm, we obtain a lower limit of D of 6.4×10^{-9} cm^2/s, assuming a collisional efficiency of 100%.

In a systematic approach to the question of the role of diffusion in mitochondrial electron transfer, we have investigated the kinetics of ubiquinol cytochrome *c* reductase in order to detect a possible diffusion limited step.

Diffusion limited enzymic reactions are those in which the time the substrate takes to diffuse to the active site in the enzyme is rate limiting to the entire reaction. This time imposes an upper value to the second order rate constant for enzyme substrate complex formation.[36]

In general high k_{cat}/K_m ratios, representing the minimum value of the association rate constant in the classical Michaelis-Menten scheme, are suggestive of a diffusion

TABLE 5
Summary of kinetic studies on the diffusion-limited steps in ubiquinol cytochrome *c* reductase (cf.[50,51]).

Kinetic parameter		Ubiquinol-2	Cytochrome c
k_{cat}/K_m	$M^{-1}\ s^{-1}$	7×10^6 *	5–20 × 10^7
		4×10^8	
	E_a (Kcal/mol)	5.9	1.4
	viscosity dependence	Low	High
	% diffusion-limited	4–14*	50–100
		0–12	

*With ubiquinol-1

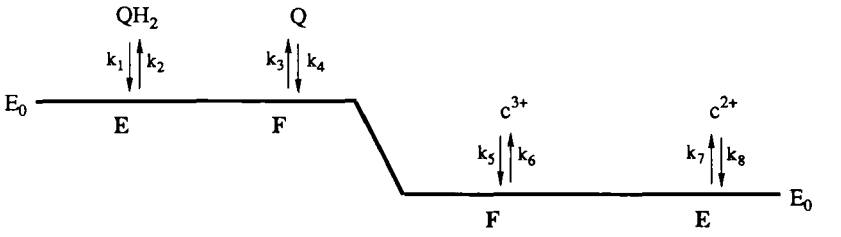

FIGURE 5 Reaction kinetic scheme of ubiquinol cytochrome c reductase (M. Degli Esposti and G. Lenaz, unpublished).

controlled rate process. Other characteristics of diffusion limited reactions are the low activation energies and the viscosity dependence of the association rate constants (or k_{cat}/K_m ratios).[36]

The results of a broad investigation on ubiquinol-cytochrome *c* reductase in submitochondrial particles are summarized in Table V. The reaction scheme of the enzyme is shown in Figure 5. The results are compatible with a diffusion limited step for the association of cytochrome *c*, but not of ubiquinol. In fact the low activation energy and the high viscosity dependence of $k_{cat}/K_{m(c)}$ (approaching k_5 in the scheme of Figure 5) are clear indications of diffusion control. On the other hand, cholesterol has little influence on the $k_{cat}/K_m(QH_2)$ (approaching k_1 in the scheme).

CONCLUSIONS

The results of this study show that electron transfer may be limited by the concentration of ubiquinone in the inner mitochondrial membrane phospholipids, which is not saturating for maximal turnovers; the upper physiological limit for CoQ concentration is probably set by the limited solubility of the quinone as a monomer in the membrane phospholipids. On the other hand, low CoQ contents, as found in some disease conditions[9] may lead to decreased electron transfer.

The hypothesis that CoQ diffusion in the lipid bilayer is also limiting for electron transfer has also been tested, but has not been found in line with the experimental results. Thus, events leading to viscosity changes of the inner mitochondrial membrane are not likely to lead to hampered electron transfer, at least as a result of lowered CoQ diffusion.

We are currently testing the hypothesis in systems where the membrane viscosity is varied *in vivo*.

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