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Localization of the Site of Oxygen Radical Generation inside the Complex I of Heart and Nonsynaptic Brain Mammalian Mitochondria

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Mitochondrial production of oxygen radicals seems to be involved in many diseases and aging. Recent studies clearly showed that a substantial part of the free radical generation of rodent mitochondria comes from complex I. It is thus important to further localize the free radical generator site within this respiratory complex. In this study, superoxide production by heart and nonsynaptic brain submitochondrial particles from up to seven mammalian species, showing different longevities, were studied under different conditions. The results, taking together, show that rotenone stimulates NADH-supported superoxide generation, confirming that complex I is a source of oxygen radicals in mammals, in general. The rotenone-stimulated NADH-supported superoxide production of the heart and nonsynaptic brain mammalian submitochondrial particles was inhibited both by *p*-chloromercuribenzoate and by ethoxyformic anhydride. These results localize the complex I oxygen radical generator between the ferricyanide and the ubiquinone reduction site, making iron–sulfur centers possible candidates, although unstable semiquinones can not be discarded. The results also indicate that the previously described inverse correlation between rates of mitochondrial oxygen radical generation and mammalian longevity operates through mechanisms dependent on the presence of intact functional mitochondria.

KEY WORDS: Mitochondria; free radicals; complex I; superoxide; iron–sulfur centers; submitochondrial particles.

INTRODUCTION

Mitochondrial free-radical production seems to be implicated in the development of aging and disease (Harman, 1972) mainly in relation to postmitotic tissues (see Barja, 1999, for review). Recent data indicate that mitochondrial mutations accumulate at high levels in cells from older humans, free radicals of mitochondrial origin being one of their more probable causes (Michikawa *et al.*, 1999). This would be also consistent with the recent observation that oxidative damage to DNA is inversely related to maximum life span of animal species in mitochondrial, but not in nuclear, DNA (Barja and Herrero, 2000). It is then important to know the precise location of the free-radical generation sites in the mitochondrial respiratory chain.

Recent studies from our laboratory have shown that not only complex III, but also complex I generates oxygen radicals in heart and nonsynaptic brain rodent mitochondria (Herrero and Barja, 1997a,b; Barja and Herrero, 1998). Complex I oxygen-radical generation seems specially important since it occurs in mitochondria from both tissues and both in the resting (state 4) and the active state (state 3), whereas complex III only generates free radicals in the heart mitochondria and only in state 4. It is then relevant to further localize the precise source of oxygen radicals inside complex I in mitochondria from these two postmitotic and vital tissues. Previous attempts to accomplish this task have

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been hampered by problems of lack of the specificity of the inhibitors used (Takeshige and Minakami, 1979; Herrero and Barja, 1997a). This is solved in this investigation by studying the effects of inhibitors acting at different points in the reaction sequence of complex I from NADH to ubiquinone under the two following simultaneous conditions: (1) the study was performed in submitochondrial particles instead of in mitochondria; (2) the effects of the inhibitors were studied in rotenone-stimulated NADH-supplemented submitochondrial particles instead of in particles supplemented with NADH alone. The study was performed on a wide range of mammalian species with different maximum life spans in order to check the validity of the conclusions to mammals, in general, and to further clarify the mechanisms responsible for the inverse correlation observed between mitochondrial free-radical generation and aging rate (Ku *et al.*, 1993; Barja 1999).

MATERIALS AND METHODS

Animals

Male rodents of the four species studied were euthanized at the laboratory by decapitation. Male sheep, pigs, cows, and horses were euthanized at the abattoir. The mean age of the animals was: 11 months (rat), 1.4 years (guinea pig), 1.5 years (rabbit), 1 year (pig), and 1.5–2.5 years (sheep, cow, and horse). The maximum longevities of the selected species are (Altman and Dittmer, 1972), 4 years (*Rattus norvegicus*), 8 years [guinea pig (*Cavia porcellus*)], 13 years [rabbit (*Oryctolagus caniculus*)], 20 years [sheep (*Ovis aries*)], 27 years [pig (*Sus scrofa*)], 30 years [cow (*Bos taurus*)], and 46 years [horse (*Equus caballus*)]. All the animals were in good health according to routine veterinary controls at the abattoir and no animal was obese or scraggy. Heart samples were taken from ventricles and brain samples were taken from occipital cortex (cow) or were whole brain (rat).

Preparation of Submitochondrial Particles

Heart mitochondria from the seven mammalian species were isolated by differential centrifugation as previously described (Herrero and Barja, 1997b). Rat and cow nonsynaptic brain mitochondria were isolated by the method of Lai and Clark (1979), which uses centrifugation in Ficoll gradients, as previously described (Barja and Herrero, 1998). Both kinds of mitochondria were resuspended in 1 ml of isolation medium (220 mM mannitol, 70 mM saccharose, 1 mM EDTA, 10 mM Tris, pH 7.4 at 5°C). These suspensions were sonicated twice over ice during 30 s with 1-min intervals between sonications, using a 50-W Vibra Cell Sonicator at setting (50–60%). After sonication, 15 ml of isolation medium were added, samples were centrifuged at 10.000 $\times$ *g* for 10 min, and the pellet discarded. The supernatant was centrifuged at 100.000 $\times$ *g* for 40 min using a Beckman Optima XL90 ultracentrifuge. Pellets were resuspended in 15 ml of isolation medium and recentrifuged at 100.000 $\times$ *g* during 40 min. The final pellets were resuspended in 0.3 ml of isolation medium, protein was assayed by Biuret, and the rest of the preparations were used for measurement of rates of $O_2^{\cdot -}$ generation.

Kinetic Measurement of $O_2^{\cdot -}$ Generation

Rates of superoxide radical generation by submitochondrial particles were measured by spectrophotometry following the oxidation of epinephrine to adrenochrome, as described by Boveris (1984). To a spectrophotometric 0.7-ml cuvette were sequentially added incubation medium (220 mM mannitol, 70 mM saccharose, 1 mM EDTA, 10 mM Tris, pH 7.4 at 37°C), submitochondrial particles (0.5 mg/ml final concentration), catalase (2 μM), epinephrine (1 mM), and NADH (100 μM), in the presence and absence of superoxide dismutase (50 U/ml). Epinephrine was added to the cuvette from a fresh 50 mM epinephrine bitartrate solution, pH 2.0, maintained over ice. The initial increase in absorbance at 480 nm was followed spectrophotometrically. Reaction rates in the presence of superoxide dismutase are subtracted from those in the absence of this enzyme in each sample. This substraction gives the superoxide dismutase-sensitive rate of adrenochrome formation, which specifically indicates the rate of $O_2^{\cdot -}$ generation. The $E = 4.0$ mM^{-1} cm^{-1} was used to convert rates of increase in absorbance at 480 nm to nanomoles of $O_2^{\cdot -}$/min/mg protein. The assay detects one adrenochrome formed per one $O_2^{\cdot -}$ produced. When inhibitors were used, they were added at the following final concentrations: 2 μM rotenone, 58 μM *p*-chloromercuribenzoate, and 416 μM ethoxyformic anhydride. At these concentrations, the effects of *p*-chloromercuribenzoate and ethoxyformic anhydride are due to inhibition of superoxide radical production by NADH + rotenone-sup-

plemented submitochondrial particles and not to effects on SOD activity.

Statistical Analyses

The statistical significance of differences in superoxide generation in a given species under different experimental conditions and between rat and cow nonsynaptic brain submitochondrial particles was studied with the Student's *t* test. Correlations between rates of superoxide production in heart submitochondrial particles and the maximum life span of the animals were studied by linear regression. The correlations were analyzed using the Pearson correlation coefficient (r) and the degree of statistical significance of the correlation (P). The 0.05 level was selected as the point of minimal statistical significance in all the analyses.

RESULTS

The rate of superoxide production of heart submitochondrial particles from seven mammalian species measured with NADH as substrate and with NADH + rotenone is shown in Fig. 1. Rotenone significantly increased the rate of superoxide production of NADH-supplemented particles in all species except in the horse in which the slightly higher values observed in the presence of rotenone did not reach statistical significance. The mean increase due to addition of rotenone in all the species showing a significant effect almost duplicated (90% increase) the rates of superoxide production observed with NADH alone. The correlation of these rates of superoxide production with the maximum life span of the different species was not significant either in the case of NADH alone ($r^2 = 0.46$; $P < 0.09$) or NADH + rotenone ($r^2 = 0.03$; $P < 0.70$).

Addition of ethoxyformic anhydride to heart submitochondrial particles supplemented with NADH + rotenone significantly decreased superoxide radical generation in all species except in the case of the pig (Fig. 2). This seems to be due to the variability observed, since ethoxyformic anhydride decreased the rate of superoxide generation in all the individual pig animals analyzed. *p*-Chloromercuribenzoate significantly decreased the rate of superoxide generation of heart submitochondrial particles supplemented with NADH + rotenone in all the species studied (Fig. 2). This inhibition depleted superoxide generation to 15%

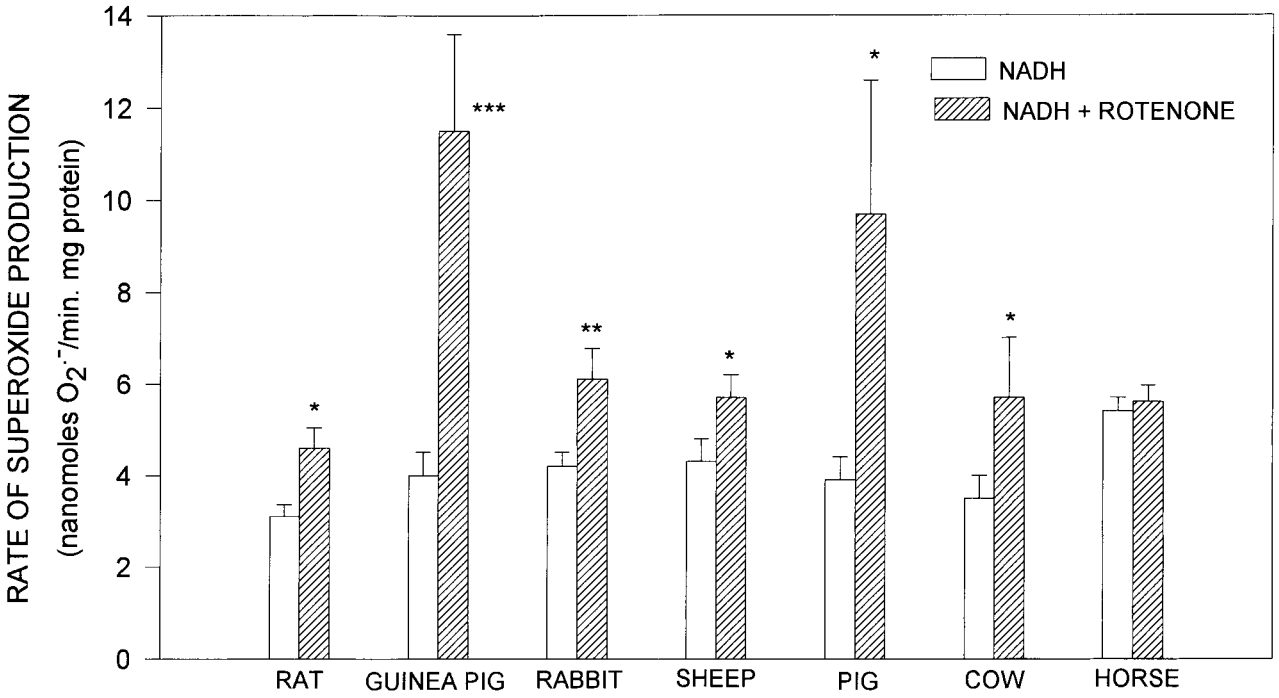

Fig. 1. Effect of rotenone on the rate of superoxide production of NADH-supplemented heart submitochondrial particles of seven mammalian species. Values are means ± SEM from four to seven animals. Asterisks describe significant differences due to addition of rotenone to NADH-supplemented particles. *, $P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

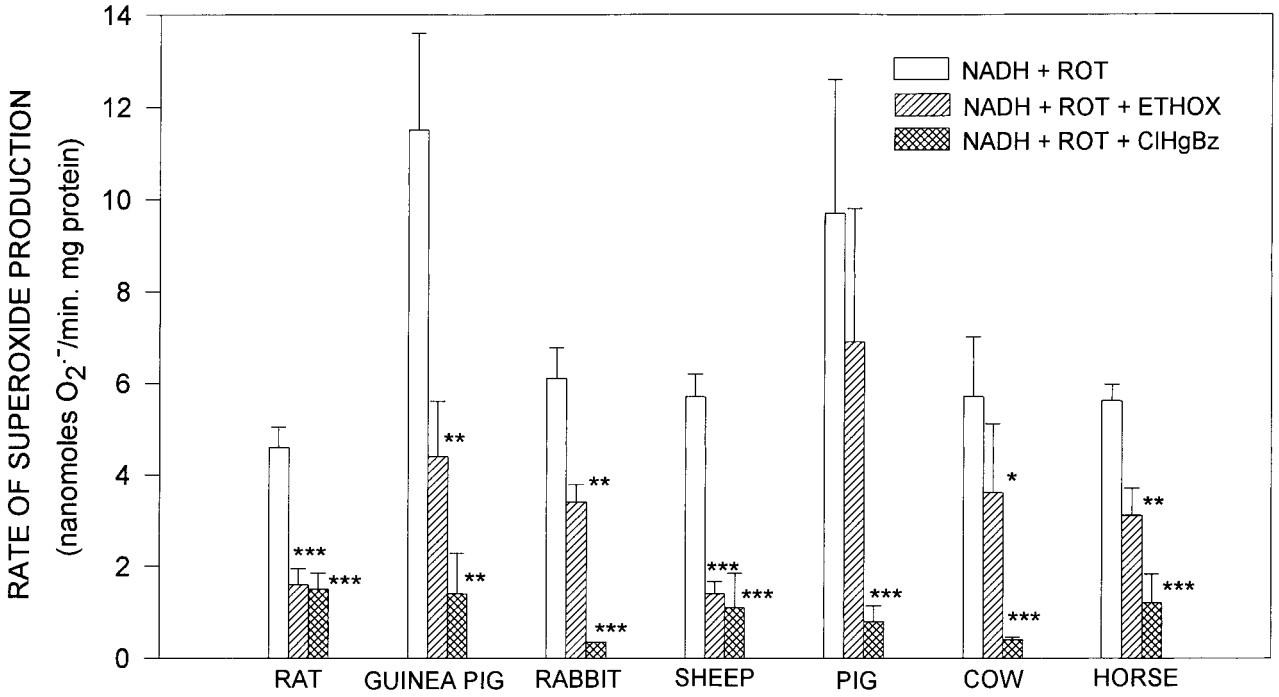

Fig. 2. Effect of addition of ethoxyformic anhydride or *p*-chloromercuribenzoate on the rate of superoxide production of heart submitochondrial particles supplemented with NADH + rotenone in seven mammalian species. Values are means from three to five animals. Asterisks describe significant differences due to addition of ethoxyformic anhydride (ETHOX) or *p*-chloromercuribenzoate (ClHgBz) to particles supplemented with NADH + rotenone (ROT). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

of the values observed with NADH + rotenone (mean depletion for all the species).

The results obtained in the heart prompted us to perform a similar study in nonsynaptic brain submitochondrial particles from rats and cows—two species showing widely different longevities (Table I). Similar to what happened in the heart, rotenone significantly increased the NADH-supported rate of superoxide radical generation by 90–110% in both species. Ethoxyformic anhydride significantly decreased superoxide production of NADH–rotenone − supplemented brain submitochondrial particles in both species (around 70% decrease). After addition of *p*-chloromercuribenzoate, the rate of superoxide generation of rat and cow brain submitochondrial particles significantly fell to 16–21% of initial values. No significant differences were found between rat and cow in any of those experimental conditions.

Table I. Rate of Superoxide Production of Nonsynaptic Brain Submitochondrial Particles in Rats and Cows under Different Conditions[a]

	Rate of superoxide production (nanomoles $O_2^{\cdot-}$/min/mg protein)			
	NADH	NADH + ROT	NADH + ROT + ETHOX	NADH + ROT + ClHgBz
Rat	2.8 ± 0.3(6)	5.2 ± 1.2(5)[b,d]	1.4 ± 0.5(6)[c,e]	1.1 ± 0.5(6)[c,e]
Cow	2.5 ± 0.3(6)	5.4 ± 1.2(5)[b,d]	1.6 ± 0.5(5)[c,e]	0.9 ± 0.7(5)[c,e]

[a] Values are means ± SEM from the number of animals shown in parentheses. No significant differences were found between rats and cows in any condition. ROT, rotenone; ETHOX, ethoxyformic anhydride; ClHgBz, *p*-chloromercuribenzoate.
[b] Significantly different from measurement with NADH.
[c] Significantly different from measurement with NADH + ROT.
[d] $P < 0.05$.
[e] $P < 0.001$.

DISCUSSION

The data obtained in this work show that complex I of mammals, in general, has capacity for oxygen radical generation. This occurs at a site situated between the ferricyanide reduction site and the rotenone-sensitive site of complex I. The results also show that the negative correlation previously observed between mitochondrial H_2O_2 production and mammalian longevity (Ku *et al.*, 1993) needs the presence of intact mitochondria for its manifestation, at least in the case of complex I, since it is not observed in submitochondrial particles.

Previous studies of mitochondrial oxygen radical production in different mammalian species have used succinate as substrate, thus mainly measuring free radicals coming from complex III of the respiratory chain. In order to analyze the possible contribution of complex I, complex I-linked substrates must be used. Initial studies showed that rotenone increases superoxide radical production of bovine heart submitochondrial particles supplemented with NADH (Turrens and Boveris, 1980). Using different combinations of substrates and specific inhibitors, we have found that complexes I and III of rat and mouse heart and complex I of rat nonsynaptic brain mitochondria produce H_2O_2 (Herrero and Barja, 1997a,b, 1998; Barja and Herrero, 1998). An important complex I oxygen radical generation has been also recently described in rat heart mitochondria (Hansford *et al.*, 1997), bovine heart mitochondria (Hasegawa *et al.*, 1997), rat brain mitochondria (Hensley *et al.*, 1998), and in isolated cardiac myocytes (Kashkarov *et al.*, 1994). Our studies showed that complex I oxygen radical generation is specially important, since it occurred in mitochondria from both tissues and both in the resting (state 4) and the active state (state 3), whereas complex III only generated free radicals in heart mitochondria and only in state 4. In the present investigation, it was shown that rotenone increased the rate of superoxide generation of NADH-supplemented heart submitochondrial particles in all the species analyzed except in the horse as well as in rat and cow nonsynaptic brain submitochondrial particles. This increase localizes the site of free-radical generation between the NADH and the rotenone-binding sites of complex I and indicates that the capacity of complex I for free-radical generation is a general characteristic of these kinds of mitochondria in mammals. This capacity is present irrespective of the longevity of the species considered, short in rodents, long in pigs and cow, and intermediate in the remainder of the species studied.

p-Chloromercuribenzoate has been previously used in studies aimed at localizing the site of oxygen radical generation within complex I (Takeshige and Minakami, 1979; Kang *et al.*, 1983; Herrero and Barja, 1997a). Those studies have limitations due to different reasons. On the one hand, it was observed that *p*-chloromercuribenzoate inhibited superoxide radical production of NADH-supplemented bovine heart submitochondrial particles (Takeshige and Minakami, 1979; Kang *et al.*, 1983). Nevertheless, these experiments can not rule out that the inhibition observed is due to the action of the thiolic agent at other respiratory complexes. This was solved in other studies by demonstrating that the inhibition by *p*-chloromercuribenzoate also occurs in pyruvate/malate plus rotenone-supplemented heart mitochondria (Herrero and Barja, 1997a), the presence of rotenone making the effect specific for complex I. However, these last experiments can not reject the possibility that *p*-chloromercuribenzoate could also act in reactions situated between the addition of substrate to the outside of mitochondria and the matrix NADH generation, like those involved in substrate transport or corresponding to mitochondrial dehydrogenases. These two problems of specificity are solved in the present investigation by performing the study in submitochondrial particles, which allow the direct addition of NADH, and in the presence of rotenone, which makes the results specific for complex I.

In all the species studied it was observed that *p*-chloromercuribenzoate inhibits superoxide generation of NADH plus rotenone heart and nonsynaptic brain submitochondrial particles. *p*-Chloromercuribenzoate is known to inhibit NADH–ubiquinone and NADH–ferricyanide reduction, but not NADH–1,2-naphtoquinone reduction at complex I (Fig. 3; Ruzicka and Crane, 1970; Ragan, 1976; Wyatt *et al.*, 1995) due to reaction with a thiol group. Since this site of inhibition is essential for NADH–ubiquinone reductase, but would not be essential for the reduction of flavin by NADH, it was proposed that the complex I oxygen radical generation would be an iron–sulfur center instead of the flavin (Takeshige and Minakami, 1979). Further localization downstream of the complex I electron path was performed here for the first time. Ethoxyformic anhydride inhibited superoxide radical production of rotenone–NADH-supplemented heart and nonsynaptic brain submitochondrial particles in almost all the species studied here. Ethoxyformic anhy-

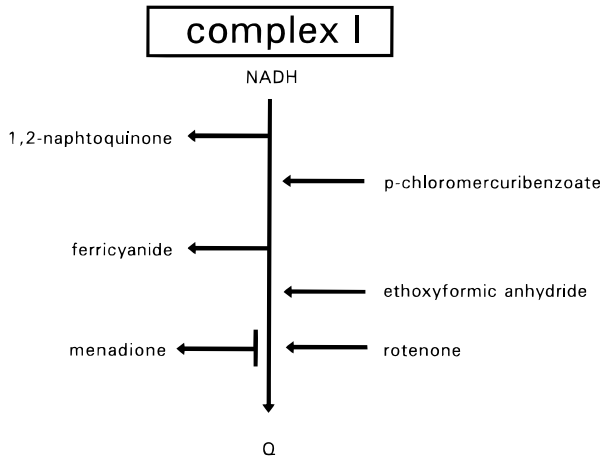

Fig. 3. The mercurial agent *p*-chloromercuribenzoate inhibits NADH–ferricyanide but not NADH-1, 2-naphthoquinone reductase in the reaction sequence from NADH to ubiquinone (Q) at complex I. Ethoxyformic anhydride inhibits NADH–menadione but not NADH–ferricyanide reductase at complex I. Based on Takeshige and Minakami (1979), Vik and Hatefi (1984), and Wyatt *et al.* (1995).

dride is known to inhibit NADH–menadione and NADH–ubiquinone, but not NADH ferricyanide reductase activities (Fig. 3; Vik and Hatefi, 1984). Thus, the complex I free-radical generator must be situated after the ferricyanide reduction site, which discards the flavin (Fig. 4) because it is situated before the site of ferricyanide reduction (Tyler, 1992; Dutton *et al.*, 1998). Due to their situation in the electron path after the ferricyanide reduction site (Fig. 4), which is based on their redox midpoint potentials, many iron–sulfur centers can be the complex I free-radical generator. Resolution of which iron–sulfur center is responsible must await the identification of their exact location in the complex I electron path. Based on the inhibition of oxygen radical production by diphenyliodonium, it has been suggested that a flavin site was involved as the complex I free-radical generator (Bailey *et al.*, 1999). However, since this compound irreversibly binds to the FMN of complex I (Li and Trush, 1998; Bailey *et al.*, 1999), it will inhibit the reduction of any free-radical generator situated between flavins and ubiquinone. Thus, the inhibition by diphenyliodonium can not discriminate between FMN and iron–sulfur centers as free-radical generators. On the other hand, although our results point to iron–sulfur clusters as oxygen radical generators, unstable semiquinones are known to be present in the membrane domain of complex I possibly functioning in H^+ pumping coupled to electron transport (Belzen *et al.*, 1997; Dutton *et al.*, 1998; Robinson, 1998). Since unstable semiquinones can reduce oxygen to superoxide, they could also be involved in complex I oxygen-radical generation. This possibility is dependent on the precise localization of these semiquinones in relation to the complex I electron pathway, which, at present, is not known.

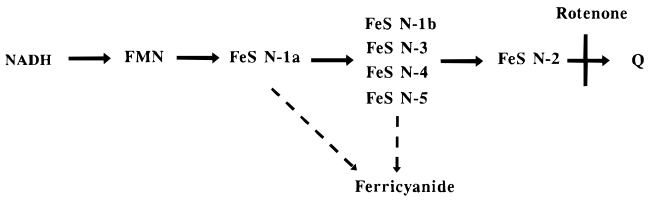

Fig. 4. Reaction scheme in complex I. The scheme is mainly based on the estimated midpoint potentials of the redox components shown. FeS are the iron–sulfur centers. The sites of inhibition by rotenone and of ferricyanide reduction are indicated. Solid arrows represent the main physiological pathway of electron transfer from NADH to ubiquinone in complex I. Based on Tyler, (1992).

Finally, the results of this investigation also showed that superoxide production of heart and nonsynaptic brain submitochondrial particles was not related to the longevity of the donor species either in the absence (NADH alone) or in the presence (stimulated rates) of rotenone. It is known, however, that H_2O_2 production of mitochondria from heart and other mammalian tissues is inversely related to longevity (Sohal *et al.*, 1990; Ku *et al.*, 1993). These two kinds of results, taken together, indicate that the mechanism, which allows longevous mammals to show low rates of mitochondrial free-radical production in relation to short-lived ones, is lost during preparation of submitochondrial particles from mitochondria. This mechanism, like respiratory control, thus needs the presence of physiologically intact mitochondria for its expression.

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