Journal of Bioenergetics and Biomembranes, Vol. 31, No. 4, 1999

Cooperation of a "Reactive Oxygen Cycle" with The Q Cycle and The Proton Cycle in the Respiratory Chain—Superoxide Generating and Cycling Mechanisms in Mitochondria

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Based on our recent findings concerning the generating, partitioning, targeting, and functioning of superoxide in mitochondria, a hypothetical model involving a "reactive oxygen cycle" in the respiratory chain has been proposed (Liu and Huang, 1991, 1996; Liu *et al.*, 1996; Liu, 1997, 1998) This model emphasizes that during State 4 respiration, an interaction between an electron leak (a branch of electron transfer directly from the respiratory chain to form $O_2^{\bullet-}$, but not H_2O) and a proton leak (a branch pathway which utilizes $\Delta\mu_{H^+}$ to produce heat, but not ATP) may take place in cooperation with the Q and proton cycles in mitochondria through the consumption of H^+ by $O_2^{\bullet-}$ anions to form a protonated perhydroxyl radical, HO_2, which is directly permeable across the inner mitochondrial membrane and induces proton leakage and a decrease of $\Delta\mu_{H^+}$. $O_2^{\bullet-}$ generation in the mitochondrial respiratory chain and its cycling across the inner membrane may have the role of an endogenous protonophore in regulating and partitioning energy transduction and heat production, as well as in pathogenesis of mitochondrial diseases, aging, and apoptosis. The present article summarizes the supporting experimental evidence obtained in this laboratory and presents a brief description of the theoretical basis of this model

KEY WORDS: Superoxide generation; protonmotive force dependent; protonophore; proton leak; heat production; ROS cycle; mitochondria.

INTRODUCTION

It is now commonly recognized that reactive oxygen species (ROS) are involved in a wide variety of pathophysiological processes, including those of AIDs, cancer, inflammation, senescence, and neurodegenerative diseases (Boiteux, 1998; Naoyuki *et. al.*, 1998; Ozawa, 1997; Sergant *et al.*, 1998). The mitochondrial respiratory chain produces 95% of superoxide radicals ($O_2^{\bullet-}$) in oxidative metabolism of the living body (Schapira, 1997). During mitochondrial respiration of State 4, a small amount of oxygen molecules can accept a single electron transfer to form $O_2^{\bullet-}$ between NADH and the site of antimycin A block. In general, only about 2–6% of oxygen consumption is partially reduced to $O_2^{\bullet-}$ (Boveris and Cadens, 1982; Chance *et al.*, 1979; Lippman, 1981) and the daily yield of $O_2^{\bullet-}$ could reach 3×10^7 molecules per mitochondria (Rochter, 1994). Some other ROS, such as $HO_2^{\bullet}$, H_2O_2, $^1O_2$, and $OH^{\bullet}$, could be formed from $O_2^{\bullet-}$. Under normal physiological conditions, the $O_2^{\bullet-}$ in the mitochondria can be metabolized by Mn^{2+}-superoxide dismutase (Mn-SOD) and other scavenging enzymes, and the steady-state concentrations of $O_2^{\bullet-}$ *in vivo* are maintained at about $10^{-11}M$ (Rochter, 1994). However, the activities of these scavenging enzymes decrease with the development of diseases and aging and the produc-

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0145-479X/99/0800-0367$16.00/0

tion of $O_2^{\cdot-}$ could be increased and accumulated in mitochondria resulting in oxidative damage of mtDNa and mitochondrial dysfunctions (Lawen *et al.*, 1994; Luft, 1994; Shigenaga *et al.*, 1994). At present, it is known that more than 100 human mitochondrial genetic diseases are related to mutations and deletions of mtDNA, in most of which, if not all, the ROS might be involved (Luft, 1994). $O_2^{\cdot-}$ and other ROS are also involved in normal cellular processes, such as cell signaling, gene expression, cell prolifiration, and differentiation, as well as programmed cell death or apoptosis. In the latter case, the mitochondrial $O_2^{\cdot-}$ and its resulting cellular redox regulation occupy a central position in the signal transduction pathway during apoptosis (Cai and Jones, 1998; Schapira, 1997; Skulachev, 1997). However, the molecular mechanism of $O_2^{\cdot-}$ generation in mitochondria and its pathophysiological functioning are not fully understood.

In this review, we summarize our recent experimental evidence concerning the generation, partitioning, and targeting of superoxide in mitochondria under different physiopathological conditions (Liu and Huang, 1991, 1996; Liu *et al.*, 1996; Liu, 1997, 1998; Zhou *et al.*, 1994). We also propose a hypothetical model involving the coexistence of a "Reactive oxygen cycle" with Q and H^+ cycles in mitochondria and emphasize that cooperation of these cycles in the respiratory chain may be essential, not only in the regulating and partitioning of energy transduction and heat production, but also in pathogenesis of mitochondrial genetic diseases, cell aging, as well as apoptosis.

SUPEROXIDE GENERATION MECHANISM IN MITOCHONDRIA

The accumulated evidence indicates that under mitochondrial State 4 respiration the univalent reduction of dioxygen may occur at different sites of the respiratory chain between NADH and the site of antimycin A block. However, the highest rates of superoxide generation (about 80%) were observed in connection with the operation of the Q cycle in complex III (Boveris and Cadens, 1982; McCord and Turrens, 1994; Rochter, 1994). Ubisemiquinone seems to serve as the primary direct electron donor, affording 80% of $O_2^{\cdot-}$ generation with the remaining 20% being derived from the NADH dehydrogenase flavoprotein (Boveris and Cadens, 1982). Since the generation of $O_2^{\cdot-}$ by the respiratory chain is a nonenzymic reaction, the rate of $O_2^{\cdot-}$ formation, as a function of the concentrations of oxygen [O_2] and of free radical ubisemiquinone [$UQH^{\cdot}$], may be represented by the following equation (Boveris and Stoppni, 1976; McCord and Turrens, 1994):

$$d[O_2^{\cdot-}]/dt = k\,[O_2]\,[UQH^{\cdot}]$$

However, our recent observation showed that in cardiac mitochondria of rat, the generation rates of $O_2^{\cdot-}$ (and H_2O_2) by succinate oxidation were increased nonlinearly with the O_2 consumption rate during State 4. The same relationship pattern was observed between the rates of cyt b_{556} reduction and State 4 respiration (Liu and Huang, 1996; Liu, 1997, 1998). To distinguish the possible roles in ROS generation between both components of the protonmotive force, $\Delta\Psi$ and ΔpH, we also compared the steady-state rates of $O_2^{\cdot-}$ and H_2O_2 generation as well as cyt b_{566} reduction in rat heart mitochondria with different $\Delta\Psi$ titrated by succinate with malonate (Liu and Huang, 1996), as in Fig 1.

Figure 1 shows that there is a clear nonlinear relationship between respiration rate and $\Delta\Psi$, as first discovered by Nicholls (1982), and subsequently confirmed by different groups (Brand *et al.*, 1994; Murphy, 1989). Interestingly, the relationships between the changes in values of $\Delta\Psi$ across the mitochondrial membrane and the rates of $O_2^{\cdot-}$ and H_2O_2 generation, as well as the reduction of cyt b_{566}, are also nonlinear and show a pattern almost identical to that of $\Delta\Psi$ versus respiration. Both CCCP and nigericin were found to inhibit $O_2^{\cdot-}$ and H_2O_2 generation in liver mitochondria from euthyroid and hyperthyroid rats (Liu, 1997, 1998). These results give clear evidence that ROS production in mitochondria is nonohmic with $\Delta\Psi$ and protonmotive-force dependent.

It is known (Turrens *et al.*, 1985, 1997) that antimycin A inhibits Q reduction at the Q_i center of the bc_1 complex and increases cyt b_{556} reduction at the Q_0 center; myxothiazol inhibits QH_2 oxidation at the Q_0 center and prevents the formation of ubisemiquinone ($QH^{\cdot}$) and the reduction of cyt b_{566}. In our system, it was also found that antimycin A stimulates $O_2^{\cdot-}$ production and myxothiazol inhibits $O_2^{\cdot-}$ generation. In addition, SOD inhibits $O_2^{\cdot-}$ production completely. Since reduced cyt b_{566} is favorable for the formation of $QH^{\cdot}$ at the Q_0 site, both cyt b_{566} reduction and $QH^{\cdot}$ formation in the Q cycle may be the essential step for $O_2^{\cdot-}$ generation at higher values of protonmotive force (Liu, 1998; Liu and Huang, 1996).

Although we do not know the precise molecular mechanism of $O_2^{\cdot-}$ generation before the site of cyt b_{552} of the respiratory chain, especially in connection

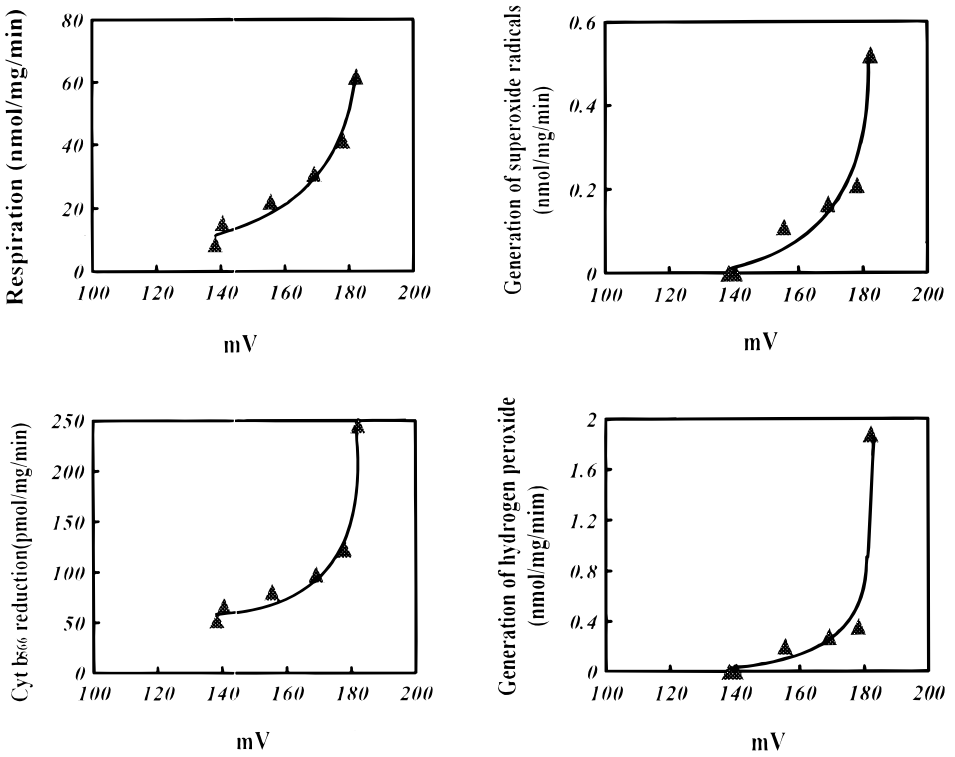

Fig. 1. Correlation between the rates of respiration, superoxide, and hydrogen peroxide generation, cytochrome b_{566} reduction and the transmembrane potential in rat heart mitochondria (from Liu, and Huang, 1996 with permission). (1) The respiration rate of mitochondria was measured using a Clark oxygen electrode on YSI Model 53 Oxygen Monitor. The reaction medium contains in 2 ml (m*M*): sucrose, 225; KCl, 20; Tris–HCl, 15, pH 7.2; KH_2PO_4, 17; $MgCl_2$ 7; BSA, 1 mg/ml; mitochondria protein, 1 mg/ml. Temperature, 25°C. Succinate at 2.5 m*M* was added to initiate the reaction. Malonate was 0–5 m*M*. (2) The transmembrane potential $\Delta\Psi$ of mitochondria was assayed fluorospectrometrically in a dialysis flow cell using rhodamine-123 as a probe. The reaction medium was 0.5 ml containing (m*M*): sucrose, 225; KCl, 10; KH_2PO_4, 10; $MgCl_2$, 5; Tris–Cl, 2, pH, 7.4; rotenone, 1 μ*M*; mitochondrial protein, 1 mg/ml; succinate, 2.5 m*M*. Rhodamine-123 (0.4–0.8 μM) was added as standard to calibrate the fluorescence intensity and the transmembrane potential, $\Delta\Psi$, was calculated according to Nernst equation. (3) Cytochrome b_{566} reduction was measured on Hitachi 557 double-wavelength spectrophotometer following the absorbance changes at 566–575 nm according to Bovers and Chance (1973), (4) Superoxide was assayed on luminometer using 2-methyl-6-(*p*-methyoxyl)-3,7-dihydroimidozol[1,2-Á]pyrazin-3-one (MCLA) as probe according to Nakano (1990.) The reaction medium (1.0 ml) contains (m*M*): sucrose, 300; Tris–HCl, 10, pH 7.5; KPO_4, 10; KCl, 10 $MgCl_2$, 5; MCLA, 4 μM; mitochondria was 0.1 mg/ml. (5) Hydrogen peroxide was determined according to Bovers and Chance (1973). The reaction medium (2 ml) contains (m*M*): sucrose, 75; mannitol, 225; Tris–HCl, pH 7.3, 20; mitochondria, 1 mg/ml; horse radish peroxidase, 1.7 μ*M*; mitochondria was 0.1 mg/ml. Succinate (5 m*M*) was added to initiate the reaction. Temperature, 25°C; The activity of the enzyme was monitored by following the absorbance changes at 417–407 nm ($\Delta\varepsilon$ m*M* = 50 m*M*-1 cm-1).

with the operation of the Q cycle, our experimental results could constitute a basis to propose that a higher value of $\Delta\Psi$ and ΔpH across the inner mitochondrial membrane may create a suitable physical condition for $O_2^{\bullet-}$ generating and partitioning (Liu, 1989, 1998; Liu and Huang, 1996; Liu *et al.*, 1996; Wei and Liu, 1995). This could be the case for several reasons. In State 4, $\Delta\mu_{H^+}$ is so high that b_{566} can not be oxidized by b_{562} and, hence, $Q^{\bullet-}$ ($QH^{\bullet}$) becomes long-lived in the Q_0 center and more readily donates a single electron to oxygen to generate $O_2^{\bullet-}$. In addition, a high value of $\Delta\Psi$ (about 200 mV) across the inner mitochondrial membrane may have a strong inducing effect on ubisemiquinone to donate an electron directly to the oxygen molecule near the C side of the membrane, but not to b_{562} at the Q_i center near the electronegative M

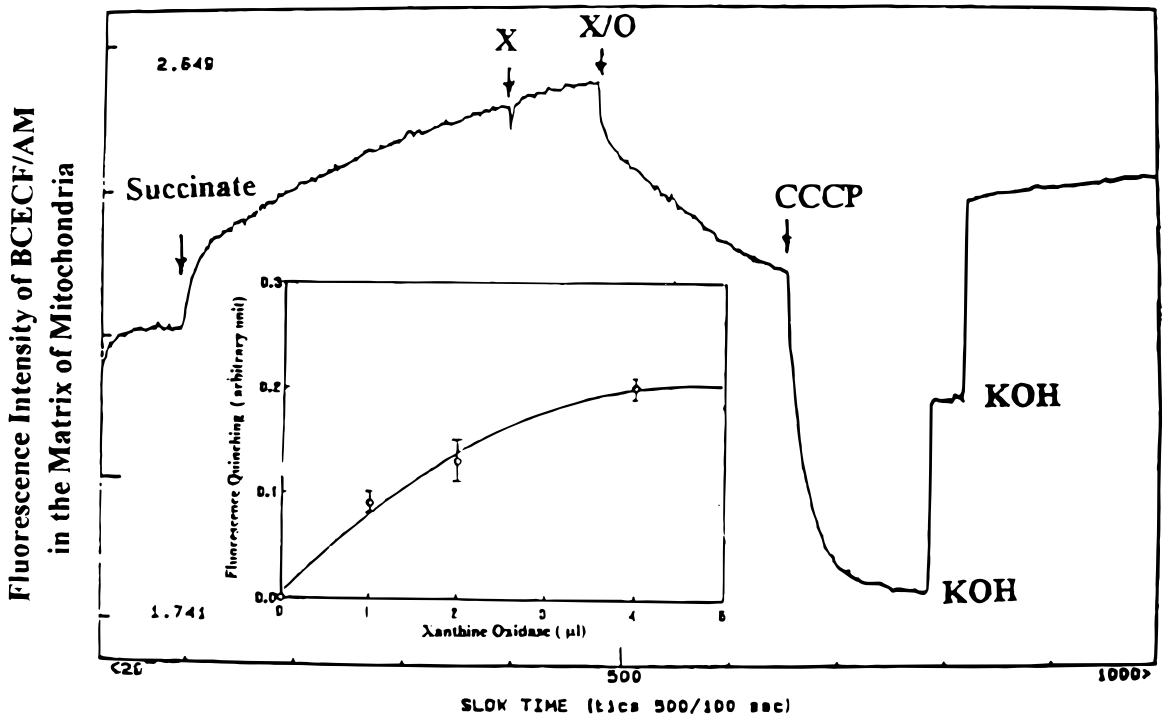

Fig. 2. Succinate oxidation increases the pH in the matrix of mitochondria and xanthine/xanthine oxidase (X/XO) decreases the pH in the matrix of mitochondria (from Liu and Huang, 1996 with permission). The reaction medium contains (m*M*): KCl, 100; K^+ - Mops, 80; pH 7.0; rotenone, 5 μ*M*; mitochondria (1 mg protein/ml) preloaded with BCECF/AM 4 μ*M*; succinate, 5 m*M*; xanthine, 240 μ*M*; xanthine oxidase, 0.028 μ*M*; CCCP, 1.0 μ*M*. The inset in Fig 2 shows that the extent of fluorescence quenching of BCECF/AM in the matrix of mitochondria was proportional to the amount of X/XO added to the medium outside of the mitochondria.

side of the inner mitochondrial membrane. In addition, there was evidence in the literature that a higher value of $\Delta\mu_H^+$ particularly of $\Delta\Psi$ in mitochondria, caused b_{566} to become reduced relative to b_{562}. The apparent midpoint potential (E_h, 7) of b_{566} changed from 40 to 0 to 70 mV (Halliward and Gutteridge, 1990). This means that a membrane potential causes the displacement of electrons from b_{562} on the matrix side to b_{566} on the cytosolic side.

Since Nicholls first found that the proton leak rates in mammalian mitochondria increased disproportionally with the increase of mitochondrial $\Delta\Psi$ (Nicholls, 1982), the increase in proton conductance across the mitochondrial membrane was also found to be nonohmic with $\Delta\Psi$ elevation (Murphy, 1989; Brand *et al.*, 1994). Our data in Fig. 1 shows that the generation of $O_2^{\cdot-}$ and H_2O_2 are nonohmic with $\Delta\Psi$, with a pattern almost identical to that of $\Delta\Psi$ versus respiration. It is logical to suggest that the increased proton conductance at high values of $\Delta\Psi$ may also be due to the higher yield of $O_2^{\cdot-}$ generation at higher values of $\Delta\Psi$ during State 4 respiration. The evidence supporting this idea also comes from our two recent reports, one of which showed that the singlet oxygen, $^1O_2$, generated in hematoporphyrin photosensization leads to an increased proton leak across the mitochondrial membrane, resulting in dissipation of $\Delta\mu_H^+$ and uncoupling of oxidative phosphorylation(Zhou *et al.*, 1994). It is known that $^1O_2$ is a group of excited oxygen molecules with strong electrophilicity and can probably be formed by superimposing the reaction of $O_2^{\cdot-}$ and H_2O_2 in biological systems (Halliward and Gutteridge, 1990). Our other paper has presented evidence that the nonlinearity between $\Delta\Psi$ and proton leak rates is also observed in rat liver mitochondria (Wei and Liu, 1995).

SUPEROXIDE INDUCES MITOCHONDRIAL PROTON LEAKAGE

P. Mitchell in 1967 first suggested that protonmotive force formed during mitochondrial respiration could have three pathways for H^+ to come back from

C side of the mitochondrial membrane to the matrix. One of methods is a proton leak pathway directly through the lipid bilayer by passive diffusion. Nicholls (1974) was the first to experimentally demonstrate that the proton leak rates in mammalian mitochondria increased disproportionally with increase mitochondrial $\Delta\Psi$. Using isolated liver cells and perfused rat skeletal musle, Brand's group was able to show that the proton leak accounts for about 26% of oxygen consumption in resting liver cells and about 52% in resting rat skeletal muscle. In addition, the experiment of Challoner provided an upper limit estimate of 12% for the contribution of proton leak to the respiration rate of the intact heart (Rolfe and Brand, 1997). These authors further emphasized that the proton leak is an important component of cellular metabolism and suggested several different physiological functions for this leak, including heat production to maintain body temperature and reduction of harmful free radical formation. However, thus far, the molecular mechanism for the proton leak in mitochondria is still unknown (Skulachev, 1996, 1997)

Recently, we found that State 4 respiration with succinate could decrease outer membrane surface pH of mitochondria or mitoplast by 0.6–1.0 pH units (Lian and Liu, 1989; Liu *et al.*, 1992). By labeling the outer surface of the mitoplast membrane with a fluorescent probe, we showed that State 4 respiration by succinate induces fluorescence quenching (a pH decrease); adding an $O_2^{\cdot-}$ generating system of xanthine/xanthine oxidase (X/XO) reversed the reaction and rapidly increased the fluorescence intensity of the probe on the mitoplast surface (a pH increase) with no simultaneous change in pH of the medium. The extent of pH increase on the mitoplast membrane surface is proportional to the amount of X/XO added (Liu and Huang, 1996)

We also observed that $O_2^{\cdot-}$ generated outside of mitochondria by X/XO could induce proton translocation from the outer surface of the mitochondrial inner membrane (C side) into the matrix (M side). The pH change in the matrix of mitochondria was detected by changes in fluorescence intensity of a soluble pH sensitive probe—BCECF/AM preloaded in the mitochondrial matrix. The pH in the matrix was rapidly increased by the proton pumping activity of succinate oxidation or ATP hydrolysis. Carboxyatractyloside, or malonate, and KCN as well as X/XO addition were found to reverse the pH increase in the mitochondrial matrix induced by ATP or succinate energization (Liu and Huang, 1996).

Incubating mitochondria with an ATP generation system to drive the H^+ pumping of ATP hydrolysis first acidifies the surface of the inner mitochondrial membrane. Adding succinate and antimycin A,

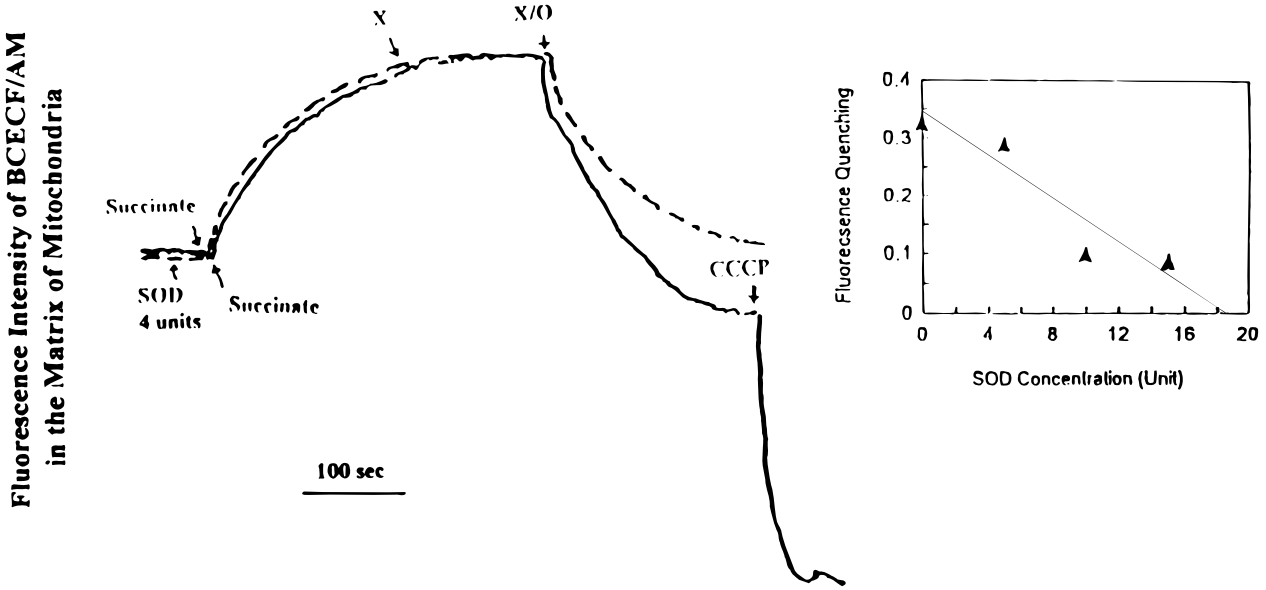

Fig. 3. The prevention effect of superoxide dismutase (SOD) on the changes in pH of the matrix of mitochondria decreased by xanthine/xanthine oxidase (X/XO) (from Liu and Huang, 1996 with permission). The condition for the experiment was the same as in Fig. 2. The extent of prevention on fluorescence quenching of BCECF/AM preloaded in the matrix of mitochondria was proportional to the amount of SOD added into the reaction medium.

(instead of X/XO), stimulates $O_2^{\cdot-}$ production probably by reducing cyt b_{566} or ubisemiquinone at the Q_o center in mitochondria and induces a significant and rapid pH decrease in the matrix of mitochondria (Liu and Huang, 1996). This set of experiments confirms that $O_2^{\cdot-}$ generated in mitochondria exhibits the same effect of decreasing pH in the matrix of mitochondria as for the exogenously generated $O_2^{\cdot-}$ system, X/XO (*in vitro* $O_2^{\cdot-}$ generation). Adding SOD to the reaction medium could prevent $O_2^{\cdot-}$ induced H^+ translocation from the outer surface of inner mitochondrial membrane into the matrix. The extent of the prevention effect of SOD is proportional to the amount of SOD added (Fig 3).

SUPEROXIDE DECREASES $H^+/2e$ RATIO AND TRANSMEMBRANE POTENTIAL ACROSS MITOCHONDRIAL MEMBRANES

From the results mentioned above, one may ask whether or not the $O_2^{\cdot-}$ generated during State 4 respiration could dissipate some sort of energy stored in the protonmotive force. The reduction of the free energy would lead to a decrease of $H^+/2e$ ratio and transmembrane potential associated with mitochondrial respiration. Our results (Liu *et al.*, 1996) indicated that the $O_2^{\cdot-}$ generating system, X/XO, decreases the energized transmembrane potential, $\Delta\Psi_E$, of mitochondria by succinate oxidation. However, the "resting" transmembrane potential, $\Delta\Psi_R$, arising from the fixed-charge differences between both surfaces of the inner mitochondrial membrane in the absence of added oxidizing substrate was not affected (Fig. 4). These results clearly confirm the idea that $O_2^{\cdot-}$ may have a specific inducing effect on proton leaks in the respiratory chain, as proposed by the authors in 1991(Liu and Huang, 1991,1996). In line with this mode of action of $O_2^{\cdot-}$ on the mitochondria, the $H^+/2e$ ratio and proton pumping activity of mitochondria oxidizing succinate were also decreased by adding X/XO(Liu *et al.*, 1996).

We have observed also that the proton leak and respiration activities in hyperthyroid mitochondria were 51 and 71% higher than those in euthyroid controls (Liu and Hu, 1995). In addition, the production of $O_2^{\cdot-}$ and respiration were 40 and 70% higher in hyperthyroid mitochondria than that of euthyroid controls. We suggested that a higher $O_2^{\cdot-}$ level in hyperthyroid animals could be the cause of a higher proton leak in the mitochondria (Liu and Hu, 1995, Liu, 1997, 1998).

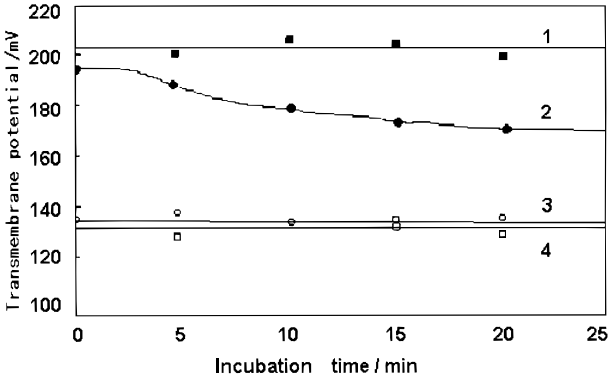

Fig. 4. Time course of effect of superoxide generated by xanthine/xanthine oxidase system on transmembrane potentials ($\Delta\Psi$) of rat cardiac mitochondria energized with or without succinate oxidation (from Liu *et al.*, 1996 with permission). Curve 1, energized mitochondrial potential ($\Delta\Psi_E$) without X/XO addition; curve 2, energized mitochondrial potential ($\Delta\Psi_E$) with X/XO addition; curve 3, nonenergized mitochondrial potential ($\Delta\Psi_R$) without X/XO addition; Curve 4, nonenergized mitochondrial potential ($\Delta\Psi_R$) with X/XO addition.

SUPEROXIDE ENHANCES HEAT PRODUCTION IN MITOCHONDRIA

Based on the results mentiond above, a question remains to be answered as to whether or not $O_2^{\cdot-}$ induces proton leakage in mitochondria leading to a decrease of protonmotive force and an increase in heat production? Mitochondria from hyperthyroid animals possess higher levels of superoxide, respiration, and proton leakage that could be used as a suitable experimental model for studying this problem (Liu and Hu, 1995).

Microcalorigraphic analysis showed that the total amount of heat production in hyperthyroid mitochondria supported by succinate (5 m*M*) was determined to be 13.03×10^{-2} J/mg mitochondrial protein and is about 70% higher than that of euthyroid mitochondria (7.67×10^{-2} J/mg mitochondrial protein). These data are consistent with the recent observation in this laboratory that State 4 respiration of hyperthyroid mitochondria is also 71% higher than that of euthyroid mitochondria (Liu and Hu, 1995). It seems that the portion of the elevated State 4 respiration in hyperthyroid mitochondria is totally associated with nonphosphorylating respiration and almost completely contributes to heat production. Interestingly, SOD could completely repress the "extra" heat production and reduces thermogenesis of hyperthyroid mitochondria to a level equal to that of euthyroid mitochondria

(7.55×10^{-2} J/mg mitochondrial protein). SOD per se had no effect on heat production. The same conclusion can be drawn from our recent observations (Table I) on heat production induced by superoxide with succinate oxidation in mitochondria from sponge and carp and rat liver (Xiao and Liu, 1998).

HYPOTHETICAL MODEL

Although the $O_2^{\cdot-}$ generation in mitochondria is usually considered a primary source of harmful oxygen-free radicals in mitochondrial disease and senescence, it may, in many situations, also have physiological significance for the bioenergetics of mitochondria as well as to cellular signaling processes. First, $O_2^{\cdot-}$ may be involved in cyanide-resistant respiration of plant and animal mitochondria, which constitutes a branch of the respiratory chain not associated with ATP formation at the level of the Q pool before the substrate side (Skulachev, 1996; Rustin, 1987; Moore and Seidow, 1991). Second, under State 4 respiration in mitochondria with high $\Delta\mu_{H^+}$, the ubisemiquinone (or reduced cyt b_{566}) formed in the Q cycle could directly donate a single electron to the oxygen molecule to make $O_2^{\cdot-}$, thus constituting an additional branch of an electron transfer pathway in connection with the Q cycle. $O_2^{\cdot-}$ can form H_2O_2 by SOD or by a self-dismutation reaction and the formed H_2O_2 can further regenerate molecular oxygen and H_2O by the action of glutathione peroxidase (Boveris and Cadens, 1982; Halliward and Gutteridge, 1990). Third, the proton leak process in mitochondria occurs only during State 4 respiration and is involved not only in regulating and partitioning energy transduction of $\Delta\mu_{H^+}$ and heat production, but could also be involved in the development of other physiopathological states (McCord and Turrens, 1994; Rolfe and Brand, 1997). In addition, ROS have been shown to be involved in a variety of cellular signaling processes as well as in apoptosis (Rusting, 1993; Kroemer *et al.*, 1995; Wachsman, 1996). In considering these points, it is logical to assume that both electron leak and proton leak processes occurring at the same location (inner mitochondrial membrane) in association with the respiratory chain and under the same conditions (State 4 respiration) should have some intrinsic connections with each other physically and/or functionally. This idea constitutes the foundation of our present hypothetical model (Liu and Huang, 1996).

Table I. Total Amount of Heat Production by Succinate Oxidation in Mitochondria from Sponge, Carp Liver, and Rat Liver[a]

Sources of mitochondria	Control	+SOD (350 U/ml)	+KCN (1 mmol/L)	+KCN and SHAM (1 mmol/L)
Sponge	690	8	13550	35.6
Carp liver	6390	410	5480	9.4
Rat liver	59	57	380	17.6

[a] Values in mJ/mg protein. By using Calvet MS-80 type of microcalorimetry, a comparative assay of the heat production of mitochondria from sponge, and carp and rat liver was carried out. Temperature, 25 °C. The reaction medium contains in 5 ml (m*M*): sucrose, 250; HEPES, 2 (pH 7.4); EDTA, 0.1; rotenone, 0.004; oligomycin, 2 mg/ml; mitochondrial protein, 1 mg/ml; succinate, 5. (From Xiao and Liu 1998).

Figure 5 summarizes the main points of our model, indicating that during State 4 respiration an interaction between electron leak (a branch of electron transfer directly from the respiratory chain to form $O_2^{\cdot-}$, but not H_2O) and proton leak (a branch pathway in utilization of $\Delta\mu_{H^+}$ to produce heat, but not ATP) may take place in cooperation with the Q and proton cycles in the mitochondrial respiratory chain.

The first postulate of the model is that under State 4 respiration a higher value of $\Delta\Psi$ (about 200 mV) across inner mitochondrial membrane may constitute a suitable physical condition, making it easier for ubisemiquinone (or reduced cyt b_{566}) to leak a single electron directly onto an oxygen molecule to produce $O_2^{\cdot-}$ at the Q_0 site of bc_1 complex facing the C side of the inner mitochondrial membrane, but not allowing donation of an electron to the cyt b_{562} at the Q_1 site near the M side of the membrane. The distance between the Q_0 site and the surface of C side of the membrane is very close (about 15Å), and the C side of the inner mitochondrial membrane is more electropositive relative to the M side. The latter is more electronegative, especially under the condition of State 4 respiration. The evidence for this model has been presented in the first set of experimental results of this article.

The second postulate of the model is that under State 4 respiration the ΔpH component of $\Delta\mu_{H^+}$ may provide a suitable chemical environment and the H^+ from the redox proton pumps could be primarily localized on the surface of the C side of the inner mitochondrial membrane, where $O_2^{\cdot-}$ may consume the protons and conduct them across the membrane to the matrix side through a pK-dependent formation of a protonated

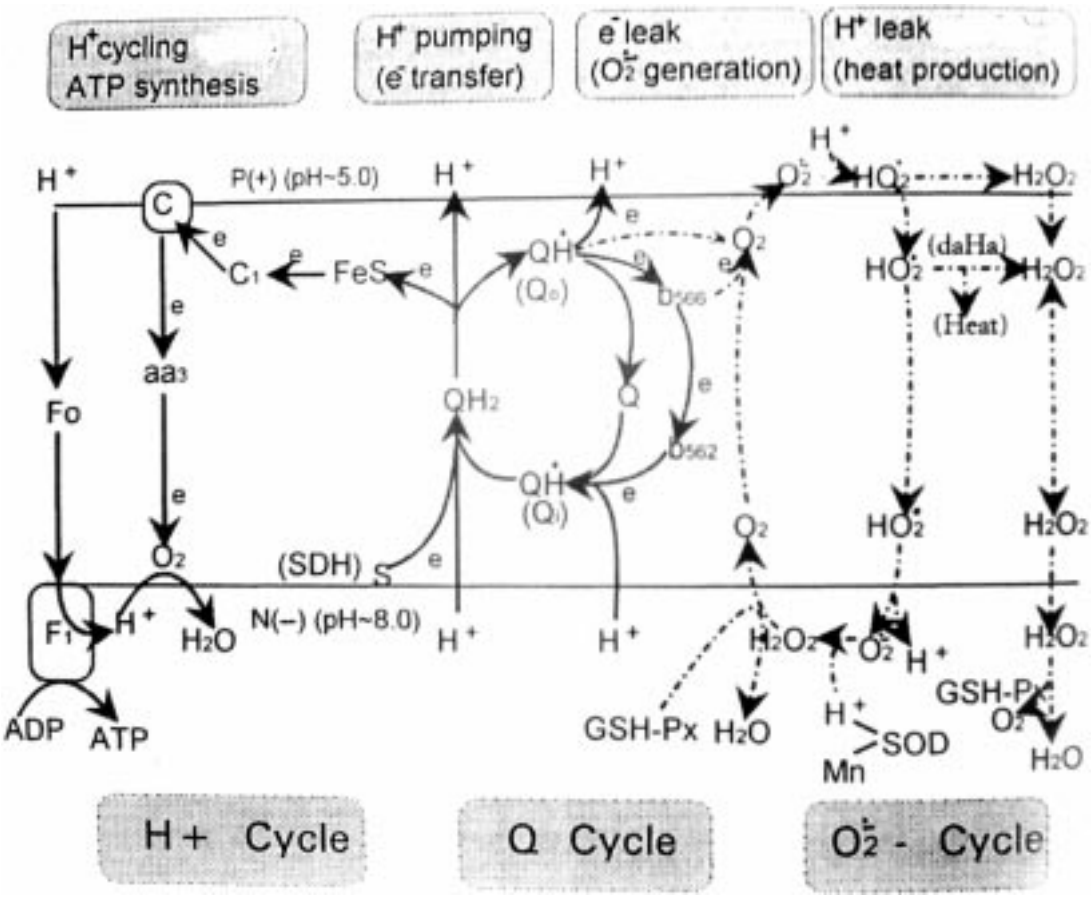

Fig. 5. A hypothetical model of coexistence of "reactive oxygen cycle" with Q and H^+ cycles in the respiratory chain of mitochondria (from Liu and Huang, 1996, with permission). For details, see text.

species of $HO_2^{\bullet}$, perhydroxyl radicals (Benon and Ross, 1985). The reaction is

$$H^+ + O_2^{\bullet -} \longleftrightarrow HO_2^{\bullet}$$

with a pK = 4.8 (Halliward and Gutteridge, 1990; Rochter, 1994;). Under acidic circumstances (for example, on the C side of the inner mitochondrial membrane under State 4 respiration, as demonstrated in our papers (Lian and Liu, 1989; Liu *et al.*, 1992; Liu, 1989; Wei and Liu, 1995), the direction of equilibrium of the reaction will be shifted toward the right side of the equation. The permeability of the neutralized $HO_2^{\bullet}$ across the membrane ought to be much higher than that of $O_2^{\bullet -}$ anions. The experimental results presented in Fig. 3 provide evidence to confirm that the second postulate may be the case. In addition, there was a report that by using an EPR assay the signal of $HO_2^{\bullet}$ can be detected in rat cardiac mitochondria under state 4 respiration (Green and Hill, 1984; Nohl, 1987).

The third postulate is that under State 4 respiration the pH in the matrix of mitochondria becomes more alkaline (the pH value is about 8.0) (Lippman, 1981) and the formed $HO_2^{\bullet}$ dissociates again into H^+ and $O_2^{\bullet -}$ anions as soon as $HO_2^{\bullet}$ is transported to the matrix. In this condition, the $\Delta\mu_{H^+}$ may also have a protective role in scavenging the $O_2^{\bullet -}$ generated during mitochondrial respiration. Usually $O_2^{\bullet -}$ is subject to the dismutation by SOD and glutathione peroxidase (GSH-Px) in the matrix of mitochondria to regenerate an oxygen molecule, which could reenter the reactive oxygen cycle as described in our model. The scheme may be represented as follows:

$$4(O_2^{\bullet -})_{out} + 4(H^+)_{out} \longrightarrow 4(HO_2^{\bullet})_{in} \qquad (1)$$

($HO_2^{\bullet}$ forms at acidic condition on the C side and diffuses across the inner mitochondrial membrane to the matrix where $[HO_2^{\bullet}]$ is low)

$$4(HO_2^{\bullet})_{in} \longrightarrow 4(O_2^{\bullet -})_{in} + 4(H^+)_{in} \quad (2)$$

($HO_2^{\bullet}$ dissociates at alkaline pH on the matrix side.)

$$4(O_2^{\bullet -})_{in} + 4(H^+)_{in} \longrightarrow 4(H_2O_2)_{in} + 2(O_2)_{in} \quad (3)$$

($O2^{\cdot -}$ dismutates under the action of Mn^{2+}-SOD in the matrix to form H_2O_2 and O_2.)

$$4(H_2O_2)_{in} \longrightarrow 2(H_2O) + O_2 \quad (4)$$

(Under the action of GSH-Px in the matrix, $2H_2O_2$ dissociate into $2H_2O$ and O_2.)
In sum,

$$4(O_2^{\bullet -})_{out} + 4(H^+)_{out} \longrightarrow 4(H_2O)_{in} + 3(O_2)_{in} \quad (5)$$

In this case, the functional role of the $O_2^{\bullet -}$ in the respiratory chain may serve as a proton carrier translocating H^+ from C to M side, just opposite to that of $QH^{\cdot}$, which brings protons from M to C side in Q cycle of respiratory chain. In addition, the H^+ regenerated in a dismutation reaction of $O_2^{\bullet -}$ in the matrix (reaction 2) can also reenter the energy-associated H^+ cycle of redox proton pumps of mitochondria. The $O_2^{\bullet -}$ in the matrix may damage mtDNA structure or the functions of mitochondria when the activity of the scavenging enzyme system in mitochondria decreases (Halliward, 1994). If $O_2^{\bullet -}$ anions in the matrix are not dismutated by Mn^{2+}-SOD, they could move out from the negatively (M side) to the positively charged compartment (C side) by facilitated electrophoresis, or be hydrolyzed and transported to C side through the $O_2^{\bullet -}$ carrier in the inner mitochondrial membrane as postulated by Skulachev (1997). According to the Skulachev scheme, the version of our scheme mentioned above will be modified as the following:

$$(O_2^{\bullet -})_{in} \longrightarrow (O_2^{\bullet -})_{out} \quad (3a)$$

(facilitated electrophoretic movement of $O_2^{\bullet -}$)

$$(O_2^{\bullet -})_{out} + (H^+)_{out} \longrightarrow (HO_2^{\bullet -})_{in} \quad (4a)$$

Alternatively, the formed $HO_2^{\bullet}$ in the membrane lipid phase may directly attack the double allylic H atoms in polyunsaturated fatty acids of membrane phospholipid of mitochondria with formation of H_2O_2, from which molecular oxygen could be regenerated (Wagner *et al.*, 1994). Certainly, further work needs to be done to determine which of the mechanisms mentioned above is more reliable. However, in this paper, we have summarized some experimental evidence from our laboratory to support the idea that $O_2^{\bullet -}$ generated in the mitochondrial respiratory chain and its cycling across the inner membrane may take place in cooperation with Q and H^+ cycles and serve as an endogenous protonophore in regulating and partitioning energy transduction and heat production.

ACKNOWLEDGMENTS

This work was supported by the China National Natural Science Foundation and The Foundation for Basic Research of Chinese Academy of Science. This work was carried out in collaboration with my various colleagues, Drs. J.P. Huang, X.H. Su, Xiao Yan, as well as Y.H. Hu, X.J.Li, and T. Xie.

REFERENCES

Benon, H. J., and Ross, A. B. (1985). *J. Phys. Chem. Ref. Data* **144**, 1041–1051.
Boiteux, S. (1998). In *Oxidative Stress in Cancer, AIDs, and Neurodegenerative Diseases*, (Montaganier, L., Oliver, R., and Pasquier, C., eds.), Marcel Dekker, New York, pp. 351–358.
Boveris, A., and Cadens, E. (1982). *Superoxide Dismutase* **11**, 15–30.
Bovers, E. C., and Chance, B. (1973). *Biochem. J.* **128**, 617–630).
Bovers, E. C., and Stoppni, A. O. M. (1976). *Biochem. J.* **156**, 453–444.
Brand, M. D., Chien, L. F., Ainscow, E. K., Rolfe, D. F. S., and Porter, R. K. (1994). *Biochem. Biophys. Acta* **1187**, 132–139.
Cai, J. Y., and Jones, D. (1998). *J. Biol. Chem.* **273**, 11401–11404.
Chance, B., Sies, H., and Boveris, A. (1979). *Physiol. Rev.* **59**, 527–605.
Green, M. J., and Hill, H. A. O. (1984). *Methods Enzymol.* **105**, 3–35.
Hillaward, B. (1994). *Nutr. Rev.* **52**, 253–265.
Hillaward, B., and Gutteridge, J. M. C. (1990). *Methods Enzymol.* **186**, 1–85.
Hillaward, B., and Gutteridge, J. M. C. (1989). *Free Radicals in Biology and Medicine*, 2nd edn., Claendon Press, Oxford, pp. 367–415.
Kroemer, G., Pett, P., Zamzami, N., Vayssiere, J. L., and Mignotte, B. (1995). *FASEB J.* **9**, 1277–1287.
Lawen, A., Martiinus, R. D., McMullen, G. L., Nagley, P., Vaillant, F., Wolvetang, E. J., and Linnane, A. W. (1994). *Mol. Aspects Med. Suppl.* **15**, s13–27.
Lian, J. P., and Liu, S. S. (1989). *Biochem. Biophys. Acta Sinica* **21**, 394–400.
Lippman, R. D. (1981). In *Bioluminescence* and *Chemiluminescence, Basic Chemistry and Analytical Application*, (DeLuca, M. A. and McElroy, W. D., eds.), Academic Press, New York, pp. 373–381.
Liu, S. S. (1989). In *Current Biochemical Research in China, A New Function of Proton Pumps in Membrane Function* (Tsou, C. L., ed.), Academic Press, New York, pp. 15–26.
Liu, S. S. (1997). *Biosci. Rep.*, **17**, 259–272.
Liu, S. S. (1998). In *Oxidative Stress in Cancer, AIDs, and Neurodegenerative Diseases* (Montaganier, L., Oliver, R., and Pasquier, C., eds.), Marcel Dekker, New York, pp. 333–349.
Liu, S. S., and Huang, J. P. (1991). In *National Conference on Free Radical Biology of China* (The Commission for Free Radical

Biology and Medicine, Chinese Biophysical Society, ed.), Beijing, p. 10.
Liu, S. S., and Huang, J. P. (1996). In *Proceedings of the International Symposium on Natural Antioxidants:Molecular Mechanisms and Health Effects* (Parker, L., Traber, M. G., and Xin, W. J., eds.), AOCS Press, Champaign, Illinois, pp, 513–529.
Liu, S. S., and Hu, Y. H. (1995). In *International Symposium on Natural Antioxidants: Molecular Mechanism and Health Effects*, 20–24 June, Beijing, China, p. 274.
Liu, S. S., Wang, X. M., Jiao, X. M., and Zhang L. (1995). *Sci. Sinica* **25**, 596–603 (in Chinese).
Liu, S. S., Wang, X. M., Jiao, X. M., and Zhang, L. (1996). *Sci. China Life Sci. Ser. C*, **39**, 168–178 (Republished in Engl.).
Liu, S. S., Xiong, J. W., and Jiao, X. M. (1992). *7th EBEG Short Rep.* **7**, 104.
Luft, R. (1994). *Proc. Natl. Acad. Sci. U.S.* **91**, 8731–8738.
McCord, J. M., and Turrens, J. F. (1994). *Current Topics Bioenerg.* **17**, 173–195.
Moore, A. L., and Seidow, J. N. (1991). *Biochem. Biophys. Acta* **1059**, 121–140
Murphy, M. P. (1989). *Biochem. Biophys. Acta* **977**, 123–141.
Nakano, M. (1990). *Methods Enzymol.* **186**, 585–591.
Naoyuki T. *et al.* (1998). In *Oxidative Stress in Cancer, AIDs, and Neurodegenerative Diseases*, (Montaganier, L., Oliver, R., and Pasquier, C., eds.), Marcel Dekker, New York, pp. 497–502.
Nicholls, D. (1982). In *Bioenergetics: An Introduction to Chemiosmotic Theory*, Academic Press, London, pp. 119–121.
Nohl, H. (1987). *FEBS Lett.* **214**, 269–273.
Ozawa, T. (1997). *Physiol. Rev.*
Rochter, C. (1994). *Current Topics Bioenerg.* **17**, 1–19.
Rolfe, D. F. S., and Brand, M. D. (1997). *Biosci. Rep.* **17**, 9–16.
Rustin, P. (1987). In *Plant Mitochondria, Structural, Functional and Physiological Aspects* (Moore A. L., and Beechy, R. B., eds.), Plenum Press, New York, pp. 37–46.
Rusting, R. (1993). *Pour la Sci.* **184**, 54–62.
Schapira, A. H. V. (1997). J. Bioenerg. Biomembr. **29**, 105–107.
Sergant, C. *et al.* (1998). In *Oxidative Stress in Cancer, AIDs, and Neurodegenerative Diseases* (Montaganier, L., Oliver, R., and Pasquier, C., eds.), Marcel Dekker, New York, pp. 409–428.
Shigenaga, M. K., Hagen, T. M., and Bruce, N. A. (1994). *Proc. Natl. Acad. Sci. U.S.* **91**, 10771–10778.
Skulachev, V. P. (1997). *Bioscie. Rep.* **17**, 347–366.
Turrens *et al.* (1982). *Arch. Biochem. Biophys.* **217**, 401–410.
Turrens *et. al.* (1985). *Arch. Biochem. Biophys.* **237**, 408–414.
Wachsman, J. T. (1996). *Mutation Res.* **350**, 25–34
Wagner, B. A., Buettner, G. R., and Burns, C. P. (1994). *Biochemistry* **33**, 4449–4453.
Wei, Y. Y., and Liu, S. S. (1995). *Chinese Biochem. J.* **11**, 593–599.
Xiao, Y., and Liu, S. S. (1998). Submitted.
Zhou, T. Q., Jiao, X. M., Wei, Y. Y., and Liu, S. S. (1994). *Progr. Natural Sci. Commun. State Key Lab. China* **4**, 313–318.