*Free Rad. Res. Comms.*, Vols. 12-13, pp. 59-66
Reprints available directly from the publisher
Photocopying permitted by license only



# SUPEROXIDE PRODUCTION BY RESPIRING MEMBRANES OF *ESCHERICHIA COLI*

JAMES A. IMLAY and IRWIN FRIDOVICH

*Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710, USA*



$O_2^-$ production by homogenates and isolated membranes of *E. coli* has been examined. Approximately one-fourth of the $O_2^-$ generated by extracts in the prescence of NAD (P) H is attributable to the membranes. The autoxidizable membrane component is a member of the respiratory chain, since $O_2^-$ production is NADH-specific, amplified by cyanide, and absent from membranes lacking the respiratory NADH dehydrogenase. Other respiratory substrates (succinate, l-phosphoglycerol, D-lactate, and L-lactate) supported $O_2^-$ production at efficiencies between 3 and 30 $O_2^-$ released per 10,000 electrons transferred, under conditions of substrate saturation.

Membranes from quinoneless mutants quantitatively retain the ability to evolve $O_2^-$, indicating that the dehydrogenases are the sites of $O_2^-$ production. Relative $O_2^-$ production was greater at low substrate concentrations, probably reflecting the facilitation of unpairing of electrons that may occur when enzymes with multiple redox centers are only partially reduced.

Respiration rate, cell volume, rates of membraneous and cytosolic $O_2^-$ production, and SOD levels were used to calculate a steady-state concentration of $O_2^-$ between $10^{-10}$ and $10^{-9}$ M in well-fed, aerobic, SOD-proficient cells.

KEY WORDS:

## INTRODUCTION

The general presence of superoxide dismutases (SODs) in aerobic organisms indicates that spontaneous $O_2^-$ formation imposes a significant oxidative stress. In fact, Touati and co-workers have established that aerobic SOD-deficient *E. coli* are only conditionally viable, apparently due to the superoxide-mediated inactivation of biosynthetic enzymes, and also mutate spontaneously at a high rate.[1,2] Similarly, yeast lacking either the cytosolic CuZnSOD or the mitochondrial MnSOD do not grow well aerobically.[3,4]

The amount, sites, and mechanisms of routine metabolic $O_2^-$ production are unknown. Three physiological sources of $O_2^-$ have been scrutinized: quinone autoxidation in mammalian mitochondria,[5] xanthine oxidase function in reperfused anoxic mammalian tissues,[6] and NADPH oxidase participation in the phagocytic respiratory burst.[7] We wished to determine the steady-state level of $O_2^-$ in unperturbed growing cells and to identify the sources which are major contributors to this level. We also wanted to establish the mechanism by which the pertinent biomolecules undergo univalent oxidation to generate $O_2^-$. Respiratory pathways were a natural focus both because of their enormous electron flux and because many respiratory components are facile at univalent redox exchange.

## METHODS

### *Preparation of Extracts*

Cells were grown in minimal salts medium containing 1% casamino acids and 0.2% glucose and were harvested in mid-log growth, washed in 50 mM KPi, pH 7.8, and lysed by passage through a French pressure cell. Inverted membrane vesicles were isolated by centrifuging the lysate 3 hours at 100,000 × g, washing, and recentrifuging. Vesicle resuspension and all subsequent assays were also in 50 mM KPi, pH 7.8.

### *Assay of $O_2^-$ Production*

Extracts or membrane preparations were incubated in the presence of reductant and 10 $\mu$M ferricytochrome *c* in a 3-ml reaction mix at 22° C. Initial rates of absorbance change were monitored on a Shimadzu UV-260 at 550 nm for 5–15 minutes. $O_2^-$ formation was calculable as the component of absorbance increase that was inhibited by the inclusion of 20 units MnSOD in the reaction mix.[8] CuZnSOD, but not heat-inactivated MnSOD, also inhibited in the manner predicted by its concentration. When included, KCN was at 3.3 mM. Absorbance changes are sufficiently slight – as little as 0.010 OD per five minutes – that machine precision limits the assay, and the error limits have been calculated accordingly. Values of $O_2^-$ production are given as nmol/min/prep, where a membrane prep consists of membranes derived from 4–8 × $10^{11}$ cells.

### *$O_2$ consumption*

Oxygen consumption was measured with a Clarke-type oxygen electrode at 22° C.

### *Dehydrogenase assay*

Dehydrogenases were assayed in terms of oxygen consumption in the presence of 3.3 mM KCN and 0.3 mM of the naphthoquinone plumbagin.

### *Strains*

The authors thank Daniele Touati for the antecedents of JI132 (*sodA sodB*),[2] I.G. Young for IY2 (*ndh*) and IY 13 (*ndh*$^+$),[9] and Robert Gennis for AN384 (*ubiA menA*) and AN387 (*ubiA*$^+$ *menA*$^+$).[10]

## RESULTS AND DISCUSSION

Superoxide generation was measured in extracts of the SOD-deficient (*sodA sodB*) strain JI132 with NADH as a reductant. Superoxide was evolved by NADH-treated cytosolic fractions at approximately ten times the rate of membrane fractions (Table I). However, superoxide generation by membranes was increased several-fold by the inclusion of cyanide in the reaction mix, presumably because cyanide blocks cytochrome oxidase and thereby enhances the steady-state reduction of components of the respiratory chain. Cytosolic superoxide production was not affected by cyanide. The

TABLE I
Superoxide Production by Subcellular Fractions (nmol per min per prep)

| | NADH | NADH + KCN |
|---|---|---|
| Cytosol | 360 ± 30 | 340 ± 30 |
| Membrane | 26 ± 8 | 100 ± 8 |

resulting 4:1 ratio of $O_2^-$ generation by cytosol compared to membranes is probably more representative of inherent autoxidation tendencies than that obtained without cyanide, since the presence of cyanide ensures that the redox-active components of both subcellular fractions are predominantly reduced: the cytosolic enzymes due to the absence of their oxidative substrates, and the respiratory membrane because interaction with its proper oxidant – oxygen – is inhibited by the cyanide.

NADPH does not contribute significantly more to superoxide evolution by either fraction. NADPH fails to elicit any superoxide production by membranes (data not shown), indicating that superoxide is evolved by the NADH-specific respiratory pathway. Cytosolic enzymes can generate $O_2^-$ in response to reduction by NADPH, but the diaphorases responsible also use NADH, since NADPH does not increase $O_2^-$ production by cytosolic fractions that are already supplied with NADH (data not shown).

Superoxide is not produced by NADH-treated membranes that lack the respiratory NADH dehydrogenase (Table II), confirming that superoxide is evolved in wild-type membranes through autoxidation of a component of the respiratory chain. This finding narrows the possible sources of NADH-dependent superoxide generation to the NADH dehydrogenase complex itself, ubiquinone, or a terminal cytochrome oxidase complex (although not at the cyanide-binding site)

Other respiratory substrates also evoke $O_2^-$ formation from membranes. Two results suggest that the $O_2^-$ must be generated by autoxidation of the dehydrogenases. First, the "leakiness" of the electron-transport chain – the $O_2^-$ formed per fixed number of electrons transferred – varies depending upon the identity of the reductant. Thus, in the membranes examined in the experiment of Table III, D-lactate generated ten times as much $O_2^-$ as did NADH per equal flux of electrons. Since all reductants employ a common pool of quinones and cytochrome oxidase complexes, the discrepancy must be due to differential rates of autoxidation of the reductant-specific dehydrogenases. Similarly, $O_2^-$ superoxide reductant evolution in the presence of cyanide – when cytochrome oxidases and quinones should be fully reduced – is quite different among reductants (Table III). For example, NADH-dependent $O_2^-$ production is much greater than that from D-lactate due to the relative preponderance of NADH dehydrogenase over D-lactate dehydrogenase in these membranes.

More directly, $O_2^-$ production per respiratory dehydrogenase is actually increased in mutants which lack respiratory quinones (Table IV). The increase is due to the block in respiratory electron outflow from the dehydrogenase and parallels the effect

TABLE II
Superoxide Generation by NADH in *ndh* Mutant Membranes (nmol per min per prep)

| | NADH | NADH + KCN |
|---|---|---|
| *ndh*$^+$ | 13 ± 4 | 23 ± 5 |
| *ndh*$^-$ | 1 ± 3 | −1 ± 3 |

TABLE III
Superoxide Production by Respiratory Substrates

| | NADH | Succinate | D-Lactate | L-Lactate | 1-Phosphoglycerol |
|---|---|---|---|---|---|
| $O_2^-$ per $10^4$ electrons | 2.8 ± 0.5 | 9.5 ± 1.4 | 31 ± 4 | 5 ± 4 | 10 ± 1.3 |
| nmol $O_2^-$ per min per prep in KCN | 166 | 56 | 64 | 11 | 190 |

of cyanide in wild-type membranes. As one would expect, cyanide has no effect on $O_2^-$ formation by the quinoneless membranes.

It thus appears that autoxidation of respiratory dehydrogenases is responsible for $O_2^-$ evolution by *E. coli* membranes. Surprisingly, the dependence of $O_2^-$ formation on substrate concentration does not match that of respiration. Autoxidation of succinate dehydrogenase (Figure 1A) is maximal at a succinate concentration of 200 $\mu$M, well beneath the respiratory $K_m$ of 860 $\mu$M; higher substrate concentrations actually suppressed $O_2^-$ production. Similar results have been obtained with NADH, D-lactate, and L-lactate. $O_2^-$ is generated by low 1-phosphoglycerol concentrations as well (Figure 1B), but no suppression was observed at high substrate concentrations.

One explanation for this suppression can be proposed on the basis of the likely site of $O_2^-$ generation: autoxidation of the flavin. All five respiratory dehydrogenases involved in this study have flavins, which are expected to be the direct recipient of electrons from the reductive substrate. Succinate dehydrogenase,[11] NADH dehydrogenase,[12] and probably 1-phosphoglycerol dehydrogenase[13] also contain iron-sulfur clusters which participate in electron transfer to quinone. D-lactate and L-lactate dehydrogenases have not yet been characterized with regard to metal content. In succinate dehydrogenase from *Bacillus subtilis*, which exhibits great structural homology to the *E. coli* enzyme, electron flow apparently occurs in the order: succinate → flavin → 2Fe-2S cluster → 3Fe-4S cluster → quinone.[14] This series also reflects the relative redox potentials in the *E. coli* enzyme.[11] Partial reduction of the enzyme, which would occur at low concentrations of reductant, would result in predominant reduction of the two univalent iron-sulfur clusters and oxidation of the flavin (Figure 2A, middle). However, the redox potential of the flavin may be sufficiently close to that of the 2Fe-2S site (-170 mV for free flavin *vs* + 10 mV for the 2Fe-2s site) that a subpopulation of half-reduced enzymes will have an alternative flavosemiquinone moiety. Full reduction of the enzyme by high substrate concentration will produce a species containing the dihydroflavoquinone (Figure 2A, bottom). Because anionic flavosemiquinones react with molecular oxygen at $\sim 10^4$ times the rate of dihydroflavoquinones,[15,16] $O_2^-$ production may be optimized by partial reduction of the enzyme and suppressed by full reduction. Divalent electron outflow to the respiratory quinone through the iron-sulfur clusters will not be suppressed.

It is also possible that occupancy of the reductant binding site by excess substrate

TABLE IV
Superoxide Production by Succinate in Quinoneless Membranes. ($O_2^-$ per succinate dehydrogenase, arbitrary units)

| Wild-Type | Wild-Type + KCN | Quinoneless | Quinoneless + KCN |
|---|---|---|---|
| 1.0 | 3.3 | 4.0 | 4.0 |

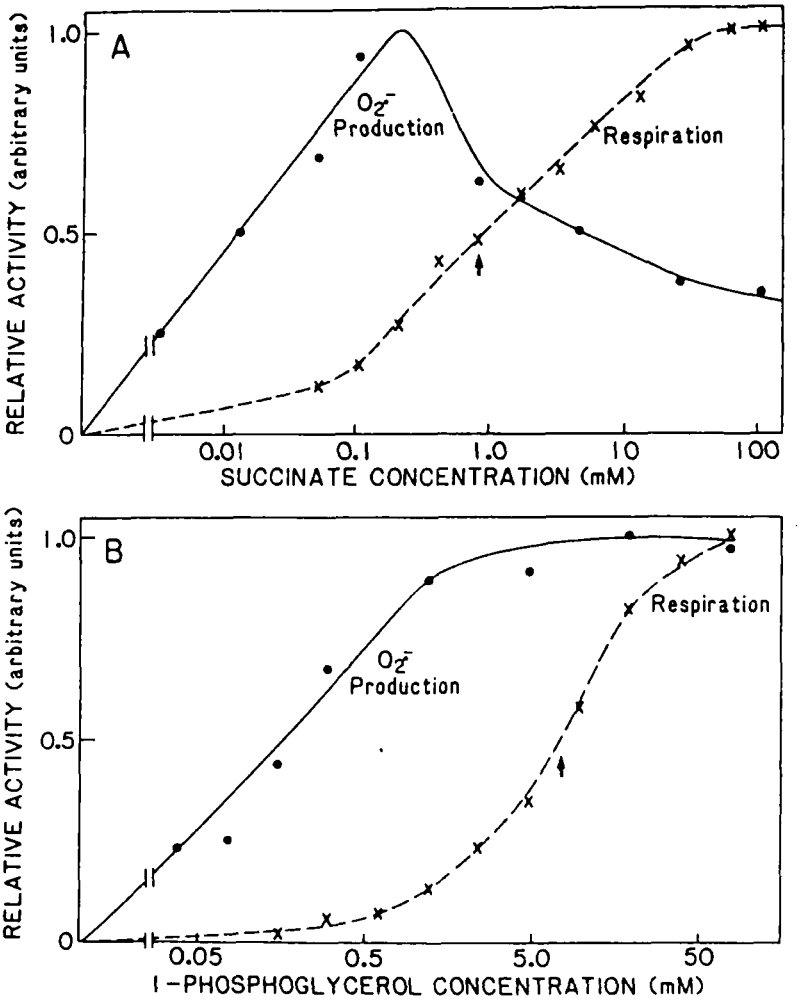


FIGURE 1 Superoxide production and respiration by membrane vesicles versus concentration of reductant. (A) Succinate. (B) 1-phosphoglycerol. Solid lines: superoxide production. Dashed lines: respiration. Arrows: $k_m$ of respiration. Values are normalized to the maxima.

sterically blocks the approach of molecular oxygen to the reduced flavin (Figure 2B). Reduction of quinones, which presumably occurs at a distinct site, would remain unhindered.

These two models for suppression of $O_2^-$ evolution by high substrate concentrations are not mutually exclusive. Both imply that $O_2^-$ formation must be a consequence of flavin autoxidation. Massey and colleagues have also noted that reduced flavins can be a source of enzymatic $O_2^-$,[17] although other workers have estimated that 80% of mitochondrial superoxide production is from quinone oxidation rather than autoxidation of dehydrogenase flavoenzymes.[5]

The optimization of dehydrogenase autoxidation by moderate substrate concentrations, which may be due to the facilitated unpairing of electron among multiple redox

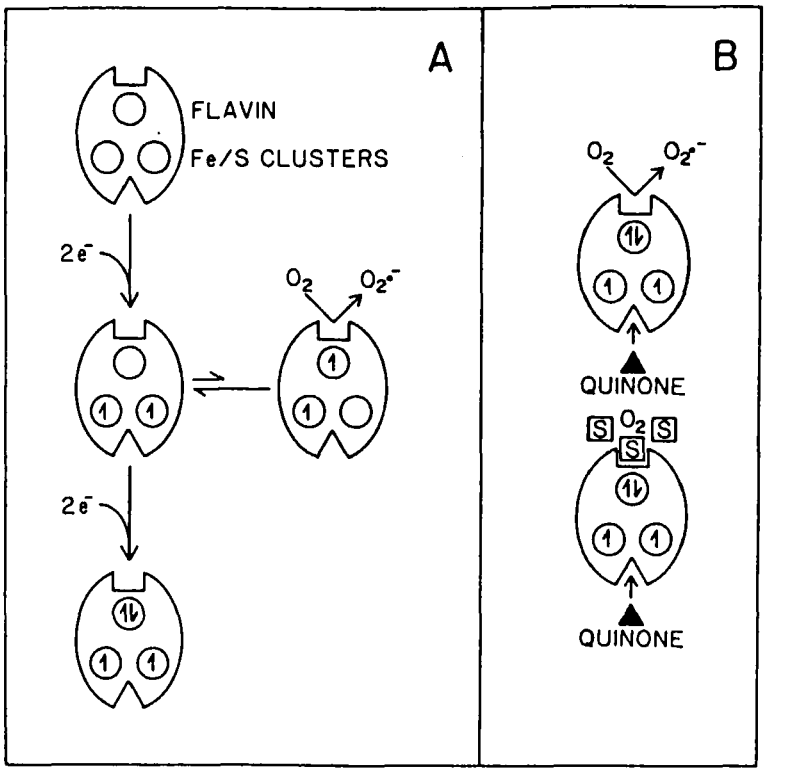


FIGURE 2 Two schemes for suppression of superoxide evolution by high concentrations of reductant. The hypothetical dehydrogenase contains two iron-sulfur clusters and a flavin. Enzyme reduction and superoxide evolution occur at the flavin; quinone (triangle) reduction occurs at a separate site through electron transfer from the iron-sulfur clusters. (A) Flavoseminquinone formation, prerequisite for superoxide evolution, occurs only in half-reduced enzymes. (B) Saturating substrate (S) blocks the approach of molecular oxygen to the flavin, site of superoxide evolution. See text for discussion.

centers, recalls the univalent oxidation of xanthine oxidase, which is suppressed when electrons fill more than two of the six available redox sites.[18] It may be generally true that significant univalent flavin oxidation can only occur when the flavosemiquinone is formed by electron sharing with other redox sites. Analogously, ubiquinone autoxidation is promoted by its binding to certain redox-active enzymes,[5] possibly because consequent electron-pair splitting between enzyme and quinone facilitates semiquinone formation.

Knowledge of $O_2^-$ generation rates by respiratory membranes allows calculation of steady-state $O_2^-$ concentrations in *E. coli*. Several bits of data are necessary. We have measured superoxide leakage from NADH-dependent respiration pathways at about 4 molecules per 10,000 electrons transferred. (This leakiness applies at physiological – *ca.* 200 $\mu$M – NADH concentrations). Each cell consumes about $7.3 \times 10^{-18}$ moles $O_2$/ sec in Luria Broth, when respiration is predominantly via NADH dehydrogenase function (unpublished observation). The cell volume, measured in this lab (by Jeff Kitzler) through comparative uptake of radiolabelled water and sucrose, is $2.8 \times 10^{-15}$ L, and each SOD-proficient cell contains $7.4 \times 10^{-9}$ units of SOD

(measured by the cytochrome *c* assay). By equating superoxide production and dismutation, one arrives at a value of $2 \times 10^{-10}$ M steady-state respiratory-produced superoxide in SOD-proficient cells. When cytosolic sources of NAD(P)H-generated superoxide are included, the estimate rises to $1 \times 10^{-9}$ M. If spontaneous dismutation were the only route of its elimination, the steady-state concentration of superoxide would be expected to reach approximately $10^{-5}$ M, or four orders of magnitude higher. (It is in fact unlikely that this last level is achieved in SOD-deficient mutants, since biomolecules other than $O_2^-$ can react with and thereby scavenge $O_2^-$).

These estimates rely on the assumption that no as yet undiscovered path of $O_2^-$ generation contributes much more than those measured above. They compare to estimates of $10^{-11}$ M made by Tyler[19] for rat liver mitochondria. Knowledge of intracellular $O_2^-$ concentrations is essential in order to relate the oxidation of biomolecules in *in vitro* test systems to the damage that might actually occur *in vivo*.



## *Acknowledgements*

This work was supported by a postdoctoral fellowship to J.A.I. from The Jane Coffin Childs Memorial Fund for Medical Research and research grants from The Council for Tobacco Research-U.S.A., Inc., the American Cancer Society, the National Science Foundation, and the National Institutes of Health.

## *References*

1. A. Carlioz and D. Touati (1986) Isolation of superoxide dismutase mutants in *Escherichia coli*: is superoxide dismutase necessary for aeorbic life? *EMBO Journal*, **5**, 623–630.
2. S.B. Farr, R. D'Ari and D. Touati (1986) Oxygen-dependent mutagenesis in *Escherichia coli* lacking superoxide dismutase. *Proceedings of the National Academy of Sciences USA*, **83**, 8268–8272.
3. T. Bilinski, Z. Krawiec, A. Liczmanski and J. Litwinska (1985) Is hydroxyl radical generated by the Fenton reaction *in vivo? Biochemical and Biophysical Research Communications*, **130**, 533–539.
4. A.P.G.M. van Loon, B. Pesold-Hurt and G. Schatz (1986) A yeast mutant lacking mitochondrial manganese-superoxide dismutase is hypersensitive to oxygen. *Proceedings of the National Academy of Sciences, USA*, **83**, 3820–3824.
5. A. Boveris and E. Cadenas (1982) Production of superoxide radicals and hydrogen peroxide in mitochondria. In *Superoxide Dismutase*, Volume II (ed. L. W. Oberly), CRC Press, Boca Raton, pp. 15–30.
6. J.M. McCord (1987) Oxygen-derived radicals: a link between reperfusion injury and inflammation. *Federal Proceedings*, **46**, 2402–2406.
7. T.R. Green and D.E. Wu (1986) The NADPH: $0_2$ oxidoreductase of human neutrophils. *Journal of Biological Chemistry*, **261**, 6010–6015.
8. J.M. McCord and I. Fridovich (1969) Superoxide dismutase, an enzymatic function for erythrocuprein. *Journal of Biological Chemistry*, **244**, 6049–6055.
9. I.G. Young and B.J. Wallace (1976) Mutations affecting the reduced nicotinamide adenine dinucleotide dehydrogenase complex of *Escherichia coli*. *Biochimica et Biophysica Acta*, **449**, 376–385.
10. D.C.-T. Au, G.N. Green and R.B. Gennis (1984) The role of quinones in the branch of the *E. coli* respiratory chain which terminates in cytochrome *o*. *Journal of Bacteriology*, **157**, 122–125.
11. C. Condon, R. Cammack, D.S. Patil and P. Owen (1985) The succinate dehydrogenase of *Escherichia coli*. *Journal of Biological Chemistry*, **260**, 9427–9434.
12. S.W. Meinharot, K. Matsushita, H.R. Kaback and T. Ohnishi (1989) EPR characterization of the iron-sulfur-containing NADH-ubiquinone oxidoreductase of the *Escherichia coli* aerobic respiratory chain. *Biochemistry*, **28**, 2153–2160.
13. A. Schryvers, E. Lohmeier and J.H. Weiner (1978) Chemical and functional properties of the native and reconstituted forms of the membrane-bound, aerobic glycerol-3-phosphate dehydrogenase of *Escherichia coli*. *Journal of Biological Chemistry*, **253**, 783–788.
14. L. Hederstedt, J.J. Maguire, A.J. Waring and T. Ohnishi. Characterization by electron paramagnetic

resonance and studies on subunit location and assembly of the iron-sulfur clusters of *Bacillus subtilis* succinate dehydrogenase. *Journal of Biological Chemistry*, **260**, 5554–5562.
15. F. Muller (1987) Flavin radicals: chemistry and biochemistry. *Free Radicals in Biological Medicine*, **3**, 215–230.
16. V. Massey, G. Palmer and D. Ballou (1973) On the reaction of reduced flavins with molecular-oxygen. In *Oxidases and Related Redox Systems* (ed. T.E. King, H.S. Mason, and M. Morrison), University Park Pess, Baltimore, pp. 25–49.
17. V. Massey, S. Strickland, S.G. Mayhew, L.G. Howell, P.C. Engel, R.G. Matthews, M. Schuman and P.A. Sullivan (1969) The production of superoxide anion radicals in the reaction of reduced flavins and flavoproteins with molecular oxygen. *Biochemical and Biophysical Research Communications*, **36**, 891–897.
18. R. Hille and V. Massey (1981) Studies on the oxidative half-reaction of xanthine oxidase. *Journal of Biological Chemistry*, **256**, 9090–9095.
19. D.D. Tyler (1975) Polarographic assay and intracellular distribution of superoxide dismutase in rat liver. *Biochemical Journal*, **147**, 493–504.

Accepted by Prof. G. Czapski