JOURNAL OF BACTERIOLOGY, Mar. 1983, p. 1282-1286
0021-9193/83/031282-05$02.00/0

Vol. 153, No. 3

Evidence for Formation of Superoxide and Formate Radicals in *Methanobacterium formicicum*

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Received 27 September 1982/Accepted 13 December 1982

Using spin labeling and spin trapping techniques in combination with electron paramagnetic resonance spectrometry, we have detected the formation of superoxide by whole cells of *Methanobacterium formicicum* under aerobic conditions in the presence and absence of sodium formate. Rates of superoxide generation have been estimated. The formation of additional free radical species, including formate, was observed. Production of these and other free radicals resulted in lipid peroxidation and concomitant cell damage.

The methanogenic bacteria are a group of strict anaerobes which can grow autotrophically using the reduction of carbon dioxide by hydrogen to produce methane (2). Only distantly related to procaryotes and eucaryotes (16), this group of organisms exhibits a marked sensitivity towards oxygen toxicity.

McCord et al. (8) initially surveyed a variety of bacterial species ranging from aerobes through aerotolerant organisms to strict anaerobes for the presence of superoxide dismutase, the enzyme which catalyzes the dismutation of superoxide to hydrogen peroxide and oxygen and which is generally considered responsible for protection against oxygen toxicity. Significant levels of superoxide dismutase were found in aerobic organisms, but very little was found in anaerobic species. Recently, Hassan and Fridovich (6) have determined that *Escherichia coli* grown anaerobically contains 17.8 U of superoxide dismutase per mg of protein. However, this enzyme has been detected in *Methanobacterium formicicum* at a level of only 2.1 U/mg of protein (T. W. Kirby, Department of Biochemistry, Duke University, Durham, N.C., personal communication). The presence of superoxide dismutase in this strict anaerobe suggests that under conditions of transient oxygen contamination, the bacterium may generate superoxide as a by-product of metabolism. In this report we demonstrate by spin labeling and spin trapping techniques that *M. formicicum* in the presence of oxygen generates superoxide, which may be responsible for cell damage. In addition, lipid peroxidation was observed as the result of the generation of other free radical species, some of which have been identified.

MATERIALS AND METHODS

Organism and growth factors. *M. formicicum* strain JF-1, isolated from sewage sludge, was grown with formate as the sole energy source and was harvested with a continuous-flow centrifuge as previously described (15). Cell paste was stored under liquid nitrogen until required, when it was suspended in a minimum volume of 50 mM bicine buffer containing 1 mM EDTA, pH 7.7, and used immediately. Under anaerobic conditions, intact whole cells have been shown by electron paramagnetic resonance (EPR) spectrometry to be reducible by formate (M. J. Barber, L. M. Siegel, N. L. Schauer, H. D. May, and J. G. Ferry, Fed. Proc. 41:891, 1982).

Synthesis of spin labels and spin traps. OXANO (2-ethyl-2,5,5-trimethyl-3-oxazolidinoxyl) was prepared by the method of Keana et al. (7) and Rosen et al. (13). OXANOH (2-ethyl-1-hydroxy-2,5,5-trimethyl-3-oxazolidine) was prepared by bubbling a 10 mM solution of OXANO with hydrogen in the presence of the catalyst platinum for 45 min (14). The hydroxylamine (OXANOH) should be prepared fresh just before use since it is unstable when stored under reducing conditions. However, the corresponding nitroxide (OXANO) is very stable, lasting for weeks in a water solution and years in a desiccator. The spin trap DMPO (5,5-dimethyl-1-pyrroline-1-oxide) was prepared by the methods outlined by Finkelstein et al. (4). The spin trap 4-POBN (*N-tert*-butyl-α-4-pyridylnitrone-1-oxide) was obtained from Aldrich Chemical Co., Milwaukee, Wis. (Fig. 1).

Measurement of superoxide generation. The formation of superoxide was determined by the oxidation of the hydroxylamine OXANOH to the corresponding nitroxide OXANO as described by Rosen et al. (12). The rate of hydroxylamine oxidation was monitored at room temperature by measuring the appearance of the EPR signal with a standard quartz flat cell in the presence and absence of superoxide dismutase. The applied field was set at the midfield peak and scanned with time by using a Varian Associates (Palo Alto,

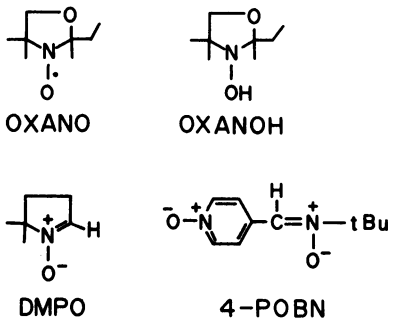

FIG. 1. Structures of spin labels and spin traps.

Calif.) model E-9 spectrometer equipped with an E-238 cavity and operating at X band.

Spin trapping of free radicals. Spin trapping of free radicals generated when *M. formicicum* was exposed to oxygen was undertaken as follows. In a typical experiment, the reaction medium contained 0.1 M spin trap, 0.1 ml of *M. formicicum* suspension, 10 mM sodium formate, and enough buffer to bring the final volume to 0.5 ml. The following necessary controls were performed, and no discernible EPR signals were detected: all spin traps were incubated with the bacterium suspension without sodium formate and with sodium formate in the absence of bacteria.

The spin trapping of lipid peroxyl radicals by 4-POBN was undertaken by using the action of lipoxygenase on linoleic acid as described by DeGroot et al. (3). The effect of 4-POBN on lipid oxidation by lipoxygenase was determined by measuring the rates of oxygen uptake with a Clarke-type electrode. Identical rates of oxygen uptake (0.14 mM/min) were obtained in the absence and presence of 4-POBN (0.1 M) from a solution of linoleic acid (sodium salt, 2.1 mM) and soybean lipoxygenase (30 μg/ml, Sigma type I) in 0.2 M CHELEX-treated sodium borate buffer at pH 9.0 containing 1 mM DETAPAC (diethylenetriamine pentaacetic acid).

The spin trapping of formate free radicals by DMPO was undertaken by photolysis (with a UV light from Fisher Scientific; model SCTI) of the model system sodium formate (10 mM) and hydrogen peroxide (1 mM) in the presence of DMPO (0.1 M).

RESULTS AND DISCUSSION

The presence of superoxide dismutase in *M. formicicum* suggests that in the presence of oxygen this bacterium may generate superoxide. Techniques for detecting this free radical vary enormously (12); however, the ability to measure superoxide generation in whole-cell preparations has only recently been reported (12). The method utilizes the oxidation of dialkylhydroxylamines to the corresponding nitroxides (11). The resultant nitroxides are stable indefinitely and are readily detectable by EPR spectrometry. The stoichiometry of the reaction is one nitroxide formed per superoxide, indicating

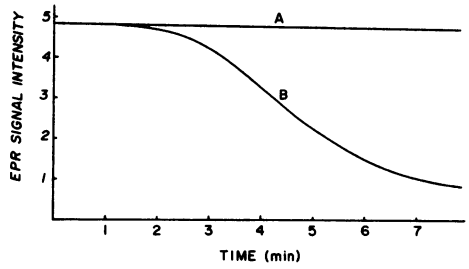

FIG. 2. Rate of OXANO elimination by *M. formicicum* in the absence (A) and presence (B) of formate (10 mM). The applied field was set at the midfield peak (3,385 G) and scanned as a function of time. Microwave power was 10 mW, and the modulation frequency was 100 kHz with an amplitude of 2 G. Sweep time was 12.5 G/min, and the receiver gain was 320 with a response time of 0.3 s.

a simple one-electron oxidation of the hydroxylamine. Since we have previously reported that *Staphylococcus aureus* reduces stable nitroxides like OXANO to the corresponding hydroxylamines (5), it is important to determine whether *M. formicicum* undergoes such an electron transfer. When OXANO was incubated with *M. formicicum*, the nitroxide was eliminated, but the rate was so slow (0.073 nmol/min per mg of protein, Fig. 2) that the ability to detect superoxide with OXANOH would not be hindered by the concurrent loss of the nitroxide. In the presence of 10 mM sodium formate, elimination of the nitroxide was rapid (with a rate of 4.6 nmol/min per mg of protein, Fig. 2); however, the elimination is preceded by approximately a 5-min lag phase (Fig. 2). These findings clearly demonstrate that the OXANOH method for the detection of superoxide can be employed if the rate of superoxide generation is so rapid that a short time period can be utilized before initiating the elimination phase. Incubation of OXANOH with *M. formicicum* aerobically in the presence

TABLE 1. Rate of superoxide generation by *M. formicicum*

Mixture	OXANOH oxidation (μM/min)[a]
Bacteria only	32.1
Bacteria + SOD[b] (1 μg/ml)	20.0
Bacteria + formate (10 mM)	49.5
Bacteria + formate (10 mM) + SOD (1 μg/ml)	39.0

[a] Cells were suspended to a protein concentration of 25 mg/ml in 50 mM bicine buffer containing 1 mM EDTA, pH 7.7. OXANOH (100 mM) was added to a solution of the 50 mM bicine buffer (final volume, 0.5 ml) containing 0.1 ml of the *M. formicicum* suspension.

[b] SOD, Superoxide dismutase.

and absence of sodium formate (10 mM) led to the rapid oxidation of OXANOH to OXANO, which was partially inhibited by superoxide dismutase (Table 1). Presumably, this oxidation by whole cells in the absence of added formate was due to the presence of residual electron donors from the culture medium. Increasing the superoxide dismutase concentration (5 μg/ml) did not diminish the rate of OXANOH oxidation. There are several possible explanations for these findings. First, the specificity of OXANOH for superoxide is not as great in *M. formicicum* as in mammalian cells, so other in situ-generated free radicals participate in this one-electron oxidation. Second, some of the superoxide generated by *M. formicicum* diffuses into the surrounding medium and is dismutated by superoxide dismutase. Although it is impossible to determine experimentally which of these explanations accounts for the partial inhibition of OXANOH oxidation by superoxide dismutase, we favor the latter hypothesis. It is now established that superoxide dismutase cannot diffuse through cell membranes (10). Based on this observation, we surmise that once superoxide is produced by *M. formicicum*, some of this free radical diffuses into the surrounding medium and reacts with superoxide dismutase in doing so.

Of interest is the observation that the rate of OXANO elimination by *M. formicicum* (Fig. 2) is markedly enhanced in the presence of sodium formate. This finding suggests that in the presence of sodium formate, *M. formicicum* generates other free radicals (e.g., formate free radical), which then promote the elimination of OXANO. To test this hypothesis, we incubated the spin trap DMPO aerobically in the presence of sodium formate (10 mM) and *M. formicicum*. The results indicate the spin trapping of two distinct radical species (Fig. 3). The presence of these two species is readily evident from changes in the line shape of the composite EPR spectrum with time. From our previous experience with spin trapping free radicals in biological systems (4), we surmised that the nature of these free radicals appeared to be due to the trapping of hydroxyl and formate radicals. To verify this hypothesis, we generated the hydroxyl and formate free radicals by chemical methods, using the photolysis of a solution containing hydrogen peroxide (1 mM) and sodium formate (10 mM) in the presence of DMPO (Fig. 4). The spectrum obtained from the model system was very similar to that observed for *M. formicicum*. These results suggest the spin trapping of hydroxyl and formate free radicals.

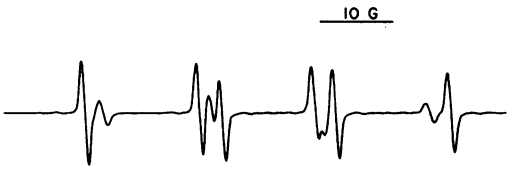

FIG. 4. Effect of formate on the spin trapping of hydroxyl radical. Hydroxyl radical was generated by the UV photolysis of hydrogen peroxide (1 mM) in 50 mM bicine buffer containing 1 mM EDTA and DMPO in the presence of formate (10 mM).

Based on these experiments, it appears that *M. formicicum* produces hydroxyl radicals via some unknown mechanism and that these radicals were spin trapped by DMPO. Alternatively, superoxide is spin trapped by DMPO (giving DMPO-OOH), which is then enzymatically reduced to DMPO-OH and observed by EPR

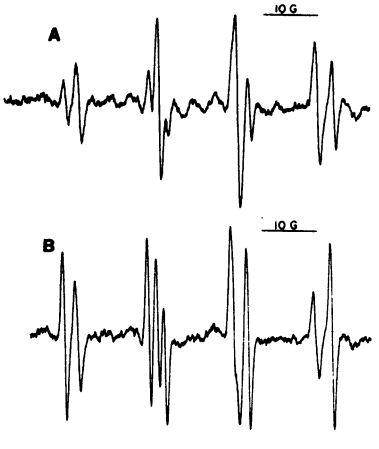

FIG. 3. EPR spectrum obtained by incubating the spin trap DMPO with *M. formicicum* in the presence of formate (10 mM). Scans were initiated 30 s (A) and 25 min (B) after the reaction had begun. Microwave power was 20 mW, and the modulation frequency was 100 kHz with an amplitude of 0.63 G. Sweep time was 12.5 G/min, and the receiver gain was 12,500 with a response time of 1 s.

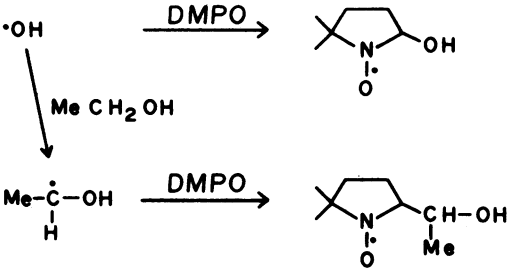

FIG. 5. Possible reaction mechanisms for hydroxyl radical in the presence of ethanol and DMPO. The hydroxyl radical can react either with ethanol, giving α-hydroxyethyl radical which can be spin trapped by DMPO, or with DMPO directly (Fig. 6).

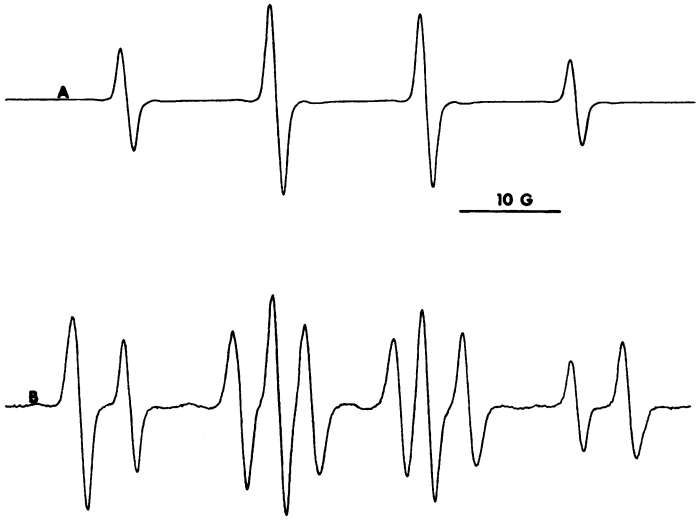

FIG. 6. Effect of ethanol on hydroxyl radical trapping by DMPO. (A) Hydroxyl radical adduct generated by UV photolysis of hydrogen peroxide; $A_H = A_N = 14.9$ G. (B) Combination of hydroxyl and α-hydroxyethyl radical adducts generated by UV photolysis of hydrogen peroxide in the presence of ethanol; $A_N = 15.8$ G, $A_H = 22.8$ G.

spectrometry. One method of verifying that hydroxyl radical trapping has taken place is to utilize the ability of spin trapping techniques to distinguish between different radical species. For example, hydroxyl radicals react with ethanol to produce hydroxyethyl radicals (1) (Fig. 5). These secondary radicals can then be spin trapped to produce an adduct with an EPR spectrum distinguishable from that of the hydroxyl adduct. Thus, if the production of DMPO-OH is due to the spin trapping of hydroxyl radicals, the addition of ethanol should both inhibit the production of DMPO-OH and result in the appearance of the α-hydroxyethyl radical (Fig. 6). It has been established that the hydroxyl radical reacts with ethanol to produce the α-hydroxyethyl radical, whereas superoxide does not undergo such a hydrogen atom abstraction (1). Since the concentration of DMPO-OH is not significantly diminished in the presence of ethanol (10 mM) (Fig. 7), it is apparent that the formation of DMPO-OH is the result of spin trapping superoxide (producing DMPO-OOH), which is then reduced by *M. formicicum*.

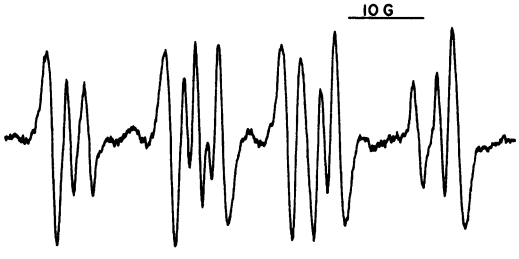

FIG. 7. EPR spectrum obtained by incubating the spin trap DMPO with *M. formicicum* in the presence of formate (10 mM) and ethanol (10 mM). Microwave power was 20 mW, and the modulation frequency was 100 kHz with an amplitude of 0.63 G. Sweep time was 12.5 G/min, and the receiver gain was 12,500 with a response time of 1 s.

With the generation of a variety of free radicals by *M. formicicum* aerobically in the presence of sodium formate, it is possible that lipid peroxidation occurs. Since lipid peroxidation is a free radical process (9), it should be possible to spin trap these radicals by using the spin trap 4-POBN. Although 4-POBN has been shown to react with both the hydroxyl and superoxide free radicals, the decomposition of these adducts is very rapid (4), and thus, these free radical species would not be detected. When *M. formicicum* was incubated aerobically with sodium formate (10 mM) in the presence of 4-POBN, we rapidly observed the spin-trapped adduct noted in Fig. 8. To confirm the identity of this spin-trapped adduct, we utilized the model system consisting of lipoxygenase-linoleic acid (3). When 4-POBN was incubated with this model system, a free radical was spin trapped which was either the linoleic acid free radical (L ·) or its hydroperoxyl analog (LOO ·). This adduct arises soon after the reaction is commenced. However, within 15 min, the oxygen concentration in the reaction medium diminished significantly, as determined by monitoring oxygen consumption with a Clarke-type electrode, and the rate of formation of the spin-trapped adduct

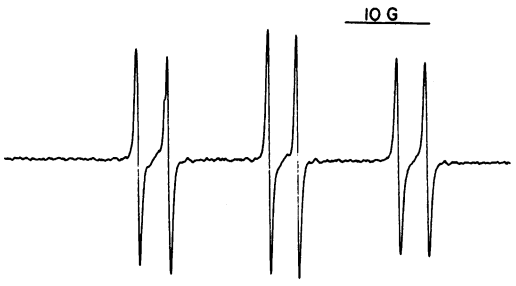

FIG. 8. EPR spectrum obtained by incubating the spin trap 4-POBN with *M. formicicum* in the presence of formate (10 mM). Microwave power was 20 mW, and the modulation frequency was 100 kHz with an amplitude of 0.63 G. Sweep time was 12.5 G/min, and the receiver gain was 1,250 with a response time of 0.3 s; A_N = 15.8 G, A_H = 3.4 G.

approximated zero. When 4-POBN was incubated with the lipoxygenase-linoleic acid model system anaerobically, no spin-trapped adduct was observed. These results identify that only lipid peroxyl radicals (LOO ·) and not lipid radicals (L ·) are spin trapped by 4-POBN. Thus, we can conclude from a series of spin-trapping experiments with model systems to verify individual species that *M. formicicum* cells incubated aerobically in the presence of sodium formate generate free radicals, including lipid radicals which initiate lipid peroxidation within the membrane structure. Finally, in the absence of sodium formate, no lipid peroxyl radicals were spin trapped by 4-POBN.

In conclusion, we have demonstrated that in the presence of oxygen, *M. formicicum* generates a variety of free radical species which include superoxide. When sodium formate is added, these bacteria oxidize this substrate to give the formate free radical, which may lead to the propagation of other free radicals, including lipid peroxyl radicals.

ACKNOWLEDGMENTS

We gratefully acknowledge the generous gift of *M. formicicum* cells from J. G. Ferry as well as timely and informative discussions.

This investigation was supported in part by Department of Defense grant DAAG-29-80-K0075.

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