Microaerobic steroid biosynthesis and the molecular fossil record of Archean life

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The power of molecular oxygen to drive many crucial biogeochemical processes, from cellular respiration to rock weathering, makes reconstructing the history of its production and accumulation a first-order question for understanding Earth's evolution. Among the various geochemical proxies for the presence of O_2 in the environment, molecular fossils offer a unique record of O_2 where it was first produced and consumed by biology: in sunlit aquatic habitats. As steroid biosynthesis requires molecular oxygen, fossil steranes have been used to draw inferences about aerobiosis in the early Precambrian. However, better quantitative constraints on the O_2 requirement of this biochemistry would clarify the implications of these molecular fossils for environmental conditions at the time of their production. Here we demonstrate that steroid biosynthesis is a microaerobic process, enabled by dissolved O_2 concentrations in the nanomolar range. We present evidence that microaerobic marine environments (where steroid biosynthesis was possible) could have been widespread and persistent for long periods of time prior to the earliest geologic and isotopic evidence for atmospheric O_2. In the late Archean, molecular oxygen likely cycled as a biogenic trace gas, much as compounds such as dimethylsulfide do today.

biomarker | photosynthesis | sterols | hypoxia | ocean

Oxygenic photosynthesis, in which water is split to provide reducing equivalents for carbon fixation, is the source of nearly all molecular oxygen on Earth. In developing the ability to produce O_2, life introduced a powerful chemical agent into the earth system and accelerated the biogenic oxidation of the surface environment. This biochemistry was apparently invented exactly once, by an ancestor of the cyanobacteria, but the specifics of how and when oxygenic photosynthesis came to be remain the subject of active investigation and debate (1–4).

The geologic record provides a minimum age for oxygenic photosynthesis. An ensemble of geological and geochemical evidence for atmospheric oxygenation, which has come to be known as the "Great Oxidation Event" (GOE), points to the period *ca.* 2.32–2.45 Ga as the time when O_2 became a persistent and geochemically significant component of the atmosphere, and prior to which atmospheric oxygen was less than 1 ppmv (4–9). Thus 2.32 Ga stands as a minimum age by which oxygenic photosynthesis must have arisen. Recently, geochemical evidence has been presented for the transient or localized presence of O_2 as much as 300 million years before the GOE (9–14), which suggests that the evolutionary origin of oxygenic photosynthesis may have significantly predated 2.32 Ga. This geologic record has given rise to divergent interpretations of the relative timing of the origin of oxygenic photosynthesis and the oxygenation of the atmosphere: Either (*i*) the GOE records both the evolutionary origin of oxygenic photosynthesis and resultant rapid atmospheric oxygenation, and apparent indicators of earlier O_2 production have been erroneously interpreted (15, 16), or (*ii*) the origin of oxygenic photosynthesis predates the GOE by at least several hundred million years, with the time gap between the two events reflecting the variety of geochemical sinks and buffers that had to be overcome before O_2 could accumulate in the atmosphere (17, 18).

Models of Archean atmospheric evolution have shown how a biogenic O_2 flux to the atmosphere could have persisted for hundreds of millions of years without causing oxygenation to an extent that would "trip" the geologic and geochemical proxies whose signals appear at or near the GOE (19–22). So long as the O_2 is accompanied by sufficient inputs of appropriate reductants (such as methane), atmospheric consumption of O_2 is rapid and there is no particular threshold value of the biogenic O_2 flux that forces the atmosphere to become oxygenated. The history of O_2 accumulation in the atmosphere is thus sensitive to variation over time in the balance between sources (oxygenic photosynthesis) and sinks (volcanic and biogenic reduced gases) of oxidizing power to the atmosphere.

The geologic and geochemical proxies that have most clearly defined the GOE are sensitive to the involvement of O_2 in weathering processes, sedimentation, and/or atmospheric chemistry and so reflect this net balance between O_2 sources and sinks. These proxies include occurrences of oxidized paleosols, detrital redox-sensitive minerals, and the mass-independent fractionation of S isotopes, as well as indices of the mobility, speciation, and/or isotopic fractionation of redox-sensitive metals (7–13, 23, 24). For signals to be recorded through these processes, molecular oxygen must have already accumulated in the atmosphere to some extent and persisted there for a geologically significant period of time. These geologic and geochemical proxies are hence somewhat removed—separated by considerations of transport and source–sink balances—from the biology of O_2 production.

Molecular Fossils and Oxygen Constraints

Hydrocarbon molecular fossils (biomarkers) have provided some of the earliest and most direct evidence for the biological production and utilization of molecular oxygen (25–30). Molecular fossils deriving from microbial membrane lipids have been found in sedimentary deposits dating as far back as 400 million years before the GOE, including in samples with the strictest contamination controls (30). Although the challenges involved in their analysis and interpretation are considerable (31, 32), authigenic molecular fossils from Archean sedimentary rocks provide a unique record of O_2 where it was first produced, accumulated, and utilized: sunlit aquatic ecosystems. As diagenetically altered yet still-identifiable biochemicals, biomarkers offer a proxy for O_2 cycling that is intimately tied to biological activity. Here our goal is to refine the inferences concerning environmental oxygenation that can be made from steranes, which are molecular fossils of

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steroids and have been detected in sedimentary rocks as old as 2.72 Ga.

Fossilized steroids have been especially significant as proxies for oxygenation, because their biosynthesis specifically requires molecular oxygen (Fig. 1). There are at least four steps in the biosynthesis of steroids that require O_2. The first O_2-dependent step is also the first committed step in steroid synthesis: the epoxidation of the linear isoprenoid squalene to produce 2,3-oxidosqualene. The enzyme that cyclizes oxidosqualene to form the characteristic steroidal 6,6,6,5-ring structure cannot act on squalene, but requires squalene (3*S*) 2,3-epoxide (33, 34).

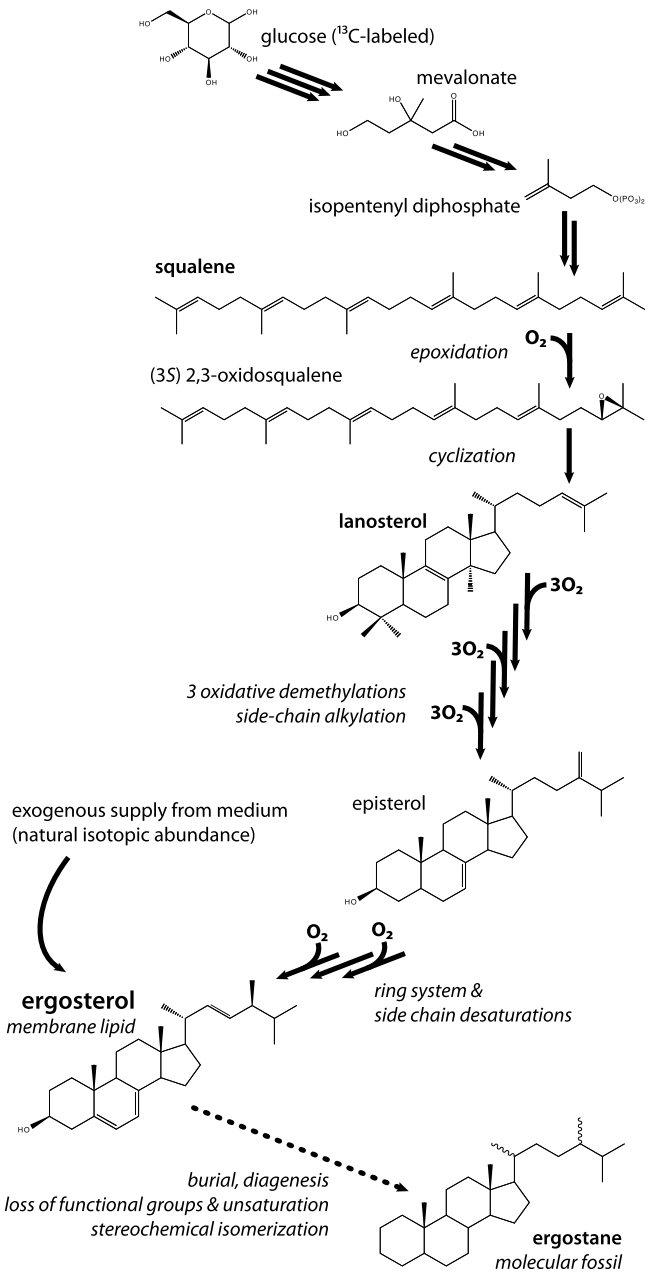

Fig. 1. A summary of the steroid biosynthetic pathway in yeast, showing the flow of carbon from glucose to ergosterol. The first oxygen-requiring step is the epoxidation of squalene; subsequently, O_2 is also required for the removal of three methyl groups from the steroid ring system and the introduction of two unsaturations in order to produce the functional membrane lipid. In these experiments, cells can either synthesize steroids de novo (and hence become labeled with ^{13}C) or take up unlabeled ergosterol from the medium. Ultimately, steroid lipids may be incorporated into sedimentary organic matter and, after diagenetic alteration, observed as sterane molecular fossils.

Subsequently, to produce the 4,14-desmethyl steroids that make up the vast majority of both membrane lipids and molecular fossils, nine more molecules of O_2 are necessary to effect three oxidative demethylation reactions. The synthesis of some steroids (e.g., cholesterol and ergosterol) requires yet more O_2 to introduce unsaturations into the carbon skeleton. These postcyclization modifications involve the chemically challenging activation of unreactive C-H bonds, in which O_2 plays an important role (35). Furthermore, these principal steps in steroid biosynthesis are conserved across some of the deepest phylogenetic divisions in the eukaryotic domain, suggesting that a version of the O_2-dependent pathway was present in the last common ancestral lineage of all extant eukaryotes (34, 36). Although a hypothetical anaerobic route to steroids has been discussed (37), there is no evidence that such a pathway exists today or did so in the past, and operation of the aerobic steroid biosynthesis pathway remains the most plausible explanation for the presence of fossil steranes in the geologic record (34).

The presence of steranes in late Archean rocks implies that some molecular oxygen was available in marine environments that produced the organic matter preserved in those sediments, but the implied degree of environmental oxygenation remains unclear. In order to leave a fossil record, O_2 levels must have been sufficient for steroid biosynthesis. To date, constraints on the concentration of dissolved O_2 that enables steroid biosynthesis are limited to three reports: Jahnke and Klein (38) reported an apparent oxygen K_m (half-saturation content) for yeast squalene epoxidase of 4.3 μM, and Rogers and Stewart reported apparent O_2 K_m values for cellular ergosterol contents in yeast of 0.5 μM (39) and "0.3 μM or less" (40). These reports, although suggestive of de novo steroid production, have not clearly documented the operation of the complete biosynthetic pathway under microaerobic conditions (here used to designate the range from approximately 1 nM to 1 μM dissolved O_2), which is a key link to interpretation of the molecular fossil record. The experiments presented here seek to establish whether steroid biosynthesis can proceed at such low dissolved O_2 concentrations, and thereby to more quantitatively assess consistency between the molecular fossil record and other geochemical proxies for oxygenation and models of atmospheric evolution.

To directly assay the O_2 requirements of steroid biosynthesis, we adopted an isotopic labeling strategy. Yeast (*Saccharomyces cerevisiae*) was chosen as a test organism because it is a facultatively anaerobic eukaryote, able to grow at any O_2 concentration in an appropriate medium. Although *S. cerevisiae* is likely only distantly related to, and certainly incompletely representative of, eukaryotic life of the late Archean, the antiquity and conservation of the core of steroid biosynthesis across the eukaryotic domain make it a useful model. Yeast cells were grown in a defined minimal medium containing ^{13}C-glucose (99 atom%) and unlabeled ergosterol (i.e., sterol with ^{13}C at the natural abundance of 1.1%). Because O_2 is essential for steroid biosynthesis, the cells are obligately auxotrophic for steroids under anaerobic conditions and take up the supplied (unlabeled) ergosterol. When O_2 is supplied to the culture, however, steroids can be made de novo from carbon substrates and thereby acquire the ^{13}C label from glucose. We grew cells at three different dissolved O_2 concentrations: 6.5 μM, 0.6 μM, and 7 nM, as well as anaerobically (<0.7 nM); O_2 levels were controlled by bubbling with defined gas mixtures ($5{,}030 \pm 100$, 473.0 ± 9.5, 5.32 ± 0.27, and <0.5 ppmv O_2 in N_2, respectively). By analyzing the cellular lipids using mass spectrometry, we could examine the incorporation of ^{13}C into specific compounds, including steroids, at the various O_2 levels.

Results

The Oxygen Requirements of Steroid Biosynthesis. The incorporation of the ^{13}C label from glucose produces a distinct shift in the appearance of lipid mass spectra (Fig. 2). Compounds that carry

abundant ^{13}C show broad, multiisotopologue peaks in their mass spectra that are shifted to higher m/z values relative to the natural abundance isotopic patterns of unlabeled compounds. These distinct spectral features provide an unambiguous signal of de novo lipid biosynthesis.

We found that the carbon-13 label is incorporated into squalene—the last steroid biosynthetic intermediate that can be produced in the absence of O_2—under all conditions (Fig. 3). Under anaerobic conditions (<0.7 nM O_2), no lanosterol is detected, and only unlabeled ergosterol (taken up from the medium) is present. This is the pattern expected if steroid synthesis is not operative and is consistent with steroid biosynthesis requiring molecular oxygen. Once O_2 is provided to the culture by bubbling, however, the observed labeling pattern changes. In the presence of O_2, lanosterol is synthesized and bears the ^{13}C label, and labeled ergosterol is also present, demonstrating de novo steroid production. Steroid biosynthesis occurred at each of the dissolved O_2 concentrations tested in our experiments: 6.5 μM, 0.6 μM, and even at 7 nM (Fig. 3)—the lowest O_2 level yet observed to enable steroid production.

We interpret these results as a useful, perhaps even conservative, lower bound for the O_2 requirement for steroid synthesis. Although yeast can serve only as a partial model for the unknown diversity of eukaryotic organisms in the Archean ocean, the fact that a modern organism can utilize such low concentrations of O_2 in biosynthesis suggests that early eukaryotes, adapted to chronic O_2 scarcity, plausibly could as well. Along with observations of oxygen-dependent fatty acid desaturation in yeasts at sub-μM O_2 (42, 43) and a recent report of microaerobic respiration by *Escherichia coli* (44), our results provide further evidence that there is a range of dissolved O_2 concentrations below the Pasteur point (of approximately 2 μM O_2, above which fermentation by many facultative anaerobes is inhibited) that enable aerobic biochemistry.

A slight shift in the labeling pattern of ergosterol is apparent between the 7 nM and the higher O_2 conditions, with the ergosterol mass spectrum in the 7-nM condition showing a larger proportion of unlabeled lipid. This suggests that cells grown with an exogenous steroid supply may shift toward a greater proportion of steroid uptake in the nanomolar O_2 range, potentially due to O_2 limitation of the rate of steroid biosynthesis. Steroid physiology in this trace-O_2 regime is likely to vary substantially between different organisms and growth conditions, making it difficult to further interpret the molecular fossil record based on these preliminary results. Nevertheless, the isotope labeling approach used in these experiments provides clear evidence of de novo steroid biosynthesis occurring at O_2 concentrations as low as 7 nM.

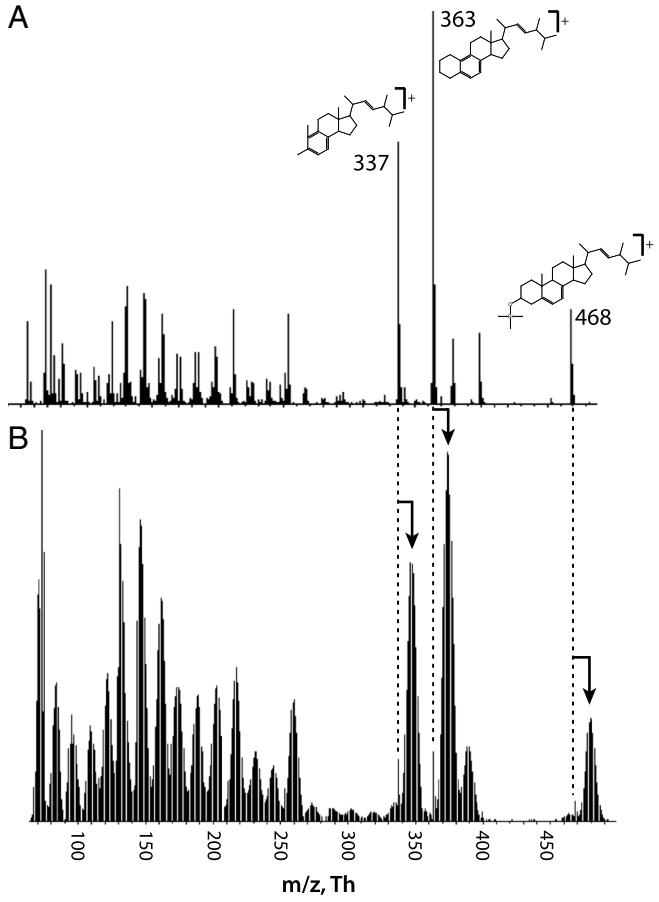

Fig. 2. Mass spectra of unlabeled (*A*) and ^{13}C-labeled (*B*) trimethylsilyl derivatives of ergosterol. Arrows indicate mass shifts of selected fragment ions resulting from incorporation of ^{13}C from labeled glucose, which produces multiisotopologue ion clusters shifted to masses higher than the unlabeled compound. Ionization was by electron impact at 70 eV; fragment ion assignments are after Brooks et al. (41)

Oxygenation of the Archean Surface Ocean. Our results demonstrate that steroid biosynthesis is possible in microaerobic environments, which has significant implications for the biogeochemical interpretation of steranes in the fossil record. The fossil steranes in late Archean sedimentary rocks can be interpreted as evidence for microaerobic conditions in a portion of the marine water column in an otherwise anoxic world. Is this interpretation of the molecular fossil record consistent with other geochemical evidence for the evolution of atmospheric O_2 levels? In particular, could microaerobic regions of the surface ocean have supplied the O_2 fluxes included in current models of Precambrian atmospheric evolution? To answer these questions, we calculated global rates of air–sea gas exchange and predicted the concentration of dissolved O_2 in the ocean surface mixed layer that is implied by given values of the O_2 flux to the atmosphere.

Under an anoxic atmosphere, the saturation level of O_2 in the ocean is very small and ingassing is negligible, so the supersaturation of O_2 in the ocean mixed layer relative to the lower atmosphere is equal to its concentration. The net sea-to-air gas flux can be expressed as

$$J_{O_2} = \alpha A_E v_p [O_2]_\alpha,$$

where J_{O_2} is the sea-to-air O_2 flux, α is the proportion of the surface area of the Earth (A_E, 510 million km^2) contributing to that O_2 flux, v_p is the globally averaged gas-exchange piston velocity, and $[O_2]_\alpha$ is the average mixed-layer O_2 concentration in the area represented by α. The gas-exchange velocity is dependent on climatic variables such as wind speed, temperature, and sea state that are poorly constrained for the Archean ocean; here we take a modern, long-term global average value of 16 cm/h (45) for the piston velocity. For a given value of the outgassing flux J_{O_2}, a low value of α (e.g., 0.01, roughly one-fifth of modern continental shelf area) represents concentration of the flux in a few oxygenated hot spots, a high value (nearer to 1) represents flux from a more globally oxygenated ocean mixed layer with a lower mean O_2 concentration.

The results of this calculation, shown in Fig. 4, indicate that the presence of microaerobic ocean surface environments, capable of supporting steroid biosynthesis, is consistent with models of Archean atmospheric evolution. Two such models, described by Pavlov et al. (21) and Zahnle et al. (22), include biogenic O_2 fluxes to the atmosphere ranging from 1×10^{11} to 3×10^{13} $cm^{-2}\,s^{-1}$; as noted above, a key feature of these models is the rapid atmospheric consumption of O_2 driven by high fluxes of reduced gases and UV radiation, which maintained the atmosphere in an anoxic state for much of the Archean.

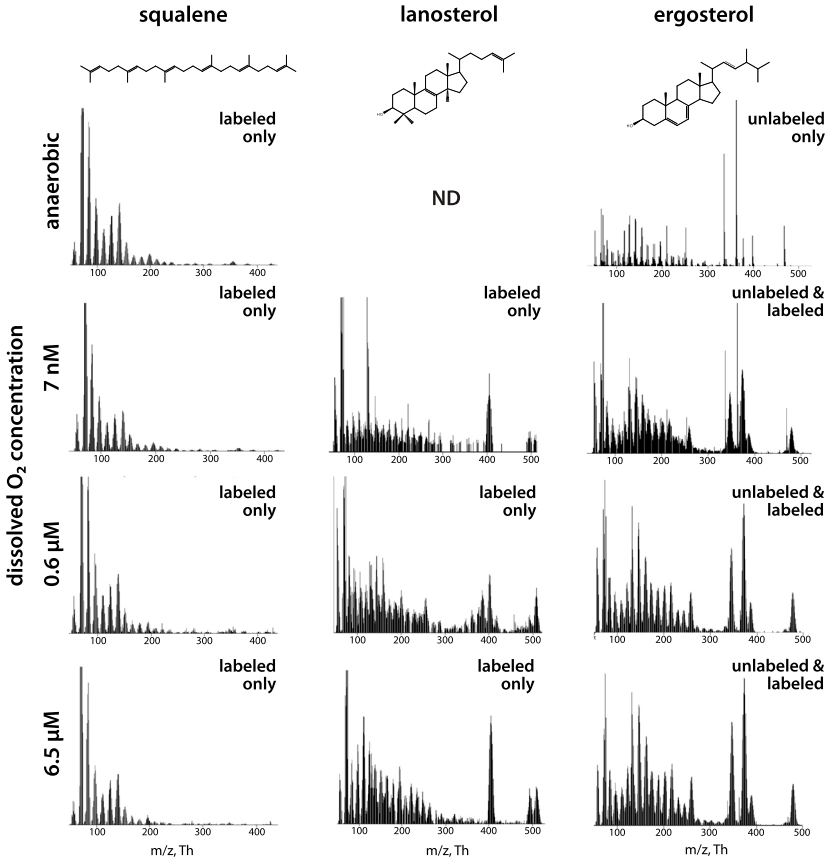

Fig. 3. Mass spectra of lipids from microaerobic growth experiments. Compounds are indicated (*top*); dissolved O_2 concentrations in the various growth conditions are indicated (*Left*). Spectra are labeled by detection of unlabeled (natural isotopic abundance; 98.9% ^{12}C) and/or isotopically labeled (multiple-^{13}C) versions of each lipid in each condition; ND, not detected. The anaerobic condition was in equilibrium with an atmosphere of <0.5 ppmv O_2, i.e., <0.7 nM dissolved O_2. Data are representative of at least two independent biological replicates for each culture condition.

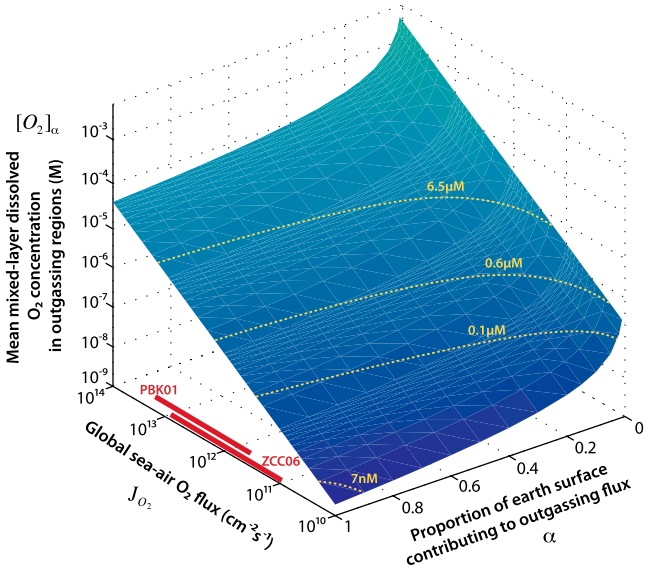

Fig. 4. The modeled average dissolved oxygen concentration in oxygenated regions of the Archean surface ocean, as a function of the sea-to-air O_2 flux and the proportion of the Earth's surface contributing to that flux. The larger the outgassing flux, and the smaller the fraction of the earth's surface it comes from, the more oxygenated surface waters in that region must be. Yellow contours indicate the dissolved oxygen concentrations of the steroid biosynthesis experiments (7 nM, 0.6 µM, and 6.5 µM) and the detection limit of the oxygen microelectrode (0.1 µM). Red bars indicate the range of O_2 fluxes in the Archean atmospheric evolution models of Pavlov et al. (21) (PBK01) and Zahnle et al. (22) (ZCC06).

Our calculations show that, to provide the O_2 outgassing fluxes described in these models, the average concentration in ocean regions contributing to the sea-to-air O_2 flux would be at least 40 nM, which is well above our experimental determination of the concentration required for steroid biosynthesis. Even if the total flux were far below the lower limit considered by Zahnle et al. (22), dissolved O_2 concentrations in higher-productivity hot spots ("oxygen oases"), would likely have been high enough to allow steroids to be made. Such hot spots probably included phototrophic microbial mats, which could have had daytime O_2 concentrations as high as 450–650 µM within the mat layers (46). The presence of microaerobic regions of the euphotic ocean by the late Archean, and their persistence over long periods of time, is consistent with geologic and geochemical evidence for an anoxic atmosphere at that time.

Discussion

We have demonstrated that steroid biosynthesis is enabled by dissolved O_2 concentrations in the nanomolar range and that the presence of such O_2 levels in the surface ocean long before 2.4 Ga is consistent with models of Archean atmospheric evolution. The molecular fossil record can thus be reconciled with geochemical proxies for the oxygenation state of the Archean surface environment, which together point to an atmosphere with <1 ppmv O_2, deep ocean waters that were often iron-rich (and therefore anaerobic), but a surface ocean that was, at least at some times and places, microaerobic. Regions of the surface ocean may have been some of the first environments on Earth to become oxygenated, and multiple lines of evidence (10–14, 29, 30) suggest that they did so hundreds of millions of years

before O_2 began to accumulate in the atmosphere to any appreciable extent.

The exceptionally low O_2 levels that enable steroid synthesis also highlight the close connection between steroid biochemistry and O_2 sensing, metabolism, and defense (47, 48). Studies of industrial fermentations have documented that, during aeration pulses, the first aerobic biochemistry to become active is steroid synthesis, even prior to respiration (49). In the experiments described here, de novo steroid synthesis occurred at nanomolar O_2 despite an abundant supply of exogenous steroids in the growth medium. It appears that steroid production is one of the most O_2-responsive biochemical systems, which is consistent with its emergence in microaerobic aquatic settings, well before the oxygenation of the atmosphere.

Steroid biosynthesis is possible at nanomolar dissolved O_2 concentrations, but biosynthesis is only the necessary first step in producing the molecular fossil record seen in late Archean rocks, which also reflects ecological and diagenetic influences. Here we have assumed that levels of O_2 sufficient for steroid biosynthesis could also be adequate to leave a range of preservable molecular fossils, but it is conceivable that the variety of sterane structures seen in late Archean sediments, and their sustained presence throughout the time period (30), points to an even higher level of environmental oxygenation. With little clear sense of the biodiversity or ecology of these early eukaryotic communities, or of the efficiency of biomarker preservation over such long time scales, it is difficult at present to move beyond the baseline constraints imposed by biosynthesis. Conversely, it could also be argued that the O_2 used to synthesize steroids in the Archean ocean was actually produced intracellularly by the eukaryotes themselves and that their presence in sedimentary organic matter reflects only the intracellular availability of O_2 rather than broader oxygenation of the marine water column. Certain modern freshwater ciliates maintain intracellular O_2 while living just below an oxic–anoxic transition zone by sequestering still-active chloroplasts from algal prey, which provide an O_2 supply for aerobic biochemistry (50). But although solely intracellular O_2 is a conceivable explanation for steroid production in isolation, all eukaryotic photosynthesis ultimately derives from cyanobacteria, through either endosymbiosis or kleptoplasty. So the presence of photosynthetic eukaryotes of any sort would mean that oxygenic cyanobacteria had already arisen and that oxygenation of aquatic habitats was under way to some extent.

In the early Precambrian, O_2 was a biogenic trace gas that was likely very rapidly cycled between dynamic sources and sinks (18). The biogeochemical distribution of O_2 in the Archean ocean probably resembled (in general terms) that of biogenic trace gases in the modern ocean, such as dimethyl sulfide (DMS). In fact, the parallels between the proposed cycling of Archean O_2 and that of modern DMS are striking: Both have biological sources and sinks in the upper water column, very low atmospheric concentrations and atmospheric lifetimes of hours to days (51, 52). The distribution of O_2 in the Archean ocean was probably geographically heterogeneous and seasonally variable, just as that of DMS is today; a recent modeling study (53) has explored spatial and temporal variability in the extremely low (approximately 10^{-8}–10^{-4} ppmv) atmospheric O_2 levels potentially generated by photochemical reactions prior to the advent oxygenic photosynthesis. In the cases of both Archean O_2 and modern DMS, some degree of supersaturation of the surface ocean is persistent over long time scales and biologically relevant, enabling uptake and utilization of the trace gas by a number of metabolic pathways.

Accurate reconstruction of the history of O_2 in the atmosphere and oceans and evolution of the organisms and biochemistry responsible for its production are central to understanding the reciprocal interactions between life and environments. In considering where and when various aerobic biochemical processes may have occurred, the past O_2 content of aquatic environments cannot be inferred from atmospheric O_2 levels alone, nor should its concentration be assumed to have been homogeneous. Biologically sustained, nanomolar-scale deviations from gas-exchange equilibria—which are invisible to many geochemical proxies for environmental oxygenation—can enable O_2-dependent metabolism, as respiration (44) and steroid biosynthesis exemplify. Recent work (53) has even suggested that, depending on the CO_2 partial pressure of the Archean atmosphere, abiotic photochemical production of O_2 and H_2O_2 might have resulted in dissolved oxygen concentrations from 10^{-15} to possibly as high as 10^{-10} molar in surface waters. Exploration of biochemistry in this subnanomolar dissolved O_2 regime, although experimentally challenging, is likely to yield further insights into the evolutionary relationships between aerobic metabolisms, oxygenic photosynthesis, and the progressive oxygenation of the surface environment.

Materials and Methods

Culture Conditions and Sampling. *S. cerevisiae* (strain D273-10B, ATCC) cells were grown in a defined minimal medium containing 4 g/L uniformly labeled $^{13}C_6$-glucose (99% ^{13}C, Cambridge Isotope), supplemented with 10 mg/L ergosterol and 0.5 ml/L Tween 80, with 1 ml/L FG-10 added as an antifoaming agent. Experiments were performed in an anaerobic chamber (Coy Scientific) with an atmosphere of 5% H_2, 15% CO_2, 80% N_2, and <1 ppmv O_2. For each growth experiment, 6 mL of late log-phase culture ($OD_{600} \sim 0.8$) was inoculated into 300 mL of media in a bubbler bottle with the headspace exhausted to external vacuum. Dissolved oxygen concentration in the medium was controlled by vigorous bubbling with either N_2 (oxygen-free grade, <0.5 ppmv O_2; Airgas) for anaerobic growth or one of three O_2:N_2 mixtures (certified O_2 contents of 5.32 ± 0.27, 473.0 ± 9.5, and $5{,}030 \pm 100$ ppmv, Airgas) for microaerobic growth. As described below, bubbling with these gas mixtures produced four dissolved O_2 levels: <0.7 nM ("anaerobic"), 7 nM, 0.6 μM, and 6.5 μM. Exponential growth rates in the four conditions were 0.24, 0.25, 0.28, and 0.29 h^{-1}, respectively. Cells were grown with bubbling and stirring for 12–16 h, until an OD_{600} of 0.2 was reached; we observed that at higher cell densities, metabolic utilization of O_2 drew down the dissolved oxygen concentration below that expected from Henry's law. The cells were then harvested by vacuum filtration inside the anaerobic chamber onto a precombusted GF/F filter. Filters were placed into glass centrifuge tubes filled with 19 mL of Bligh-Dyer (54) extraction solvent (10:5:4 chloroform:methanol:water) that had been preequilibrated with the anaerobic atmosphere and incubated in the anaerobic chamber overnight to ensure complete enzyme deactivation prior to oxygen exposure.

Dissolved O_2 Measurements. The O_2 concentration in the cultures during microaerobic growth was measured by two methods: a colorimetric assay based on Rhodazine-D (Chemetrics) and a STOX (Switchable Trace Oxygen) microelectrode with a polarizable front guard (55) (Unisense). In our experiments, the detection limits for the two methods were 300 nM for the Rhodazine-D assay and 100 nM for the STOX electrode. For the anaerobic (N_2-bubbled, <0.5 ppmv O_2) growth condition and the lowest O_2 concentration tested (5.32 ppmv O_2), the dissolved oxygen concentrations were below the detection limit of both methods and were calculated from Henry's law at <0.7 and 7 nM, respectively. Limiting the cultures to a maximum OD_{600} of 0.2 kept the rate of biological O_2 utilization below the rate at which oxygen could be supplied by bubbling. The stability of the saturation levels for the two higher dissolved O_2 levels tested—0.6 and 6.5 μM, also consistent with Henry's law for saturation with the 473 and 5,030 ppmv O_2 mixtures—during growth of the culture was verified with the STOX electrode.

Lipid Analysis. Tubes containing filters and solvent were removed from the anaerobic chamber and lipids extracted twice by ultrasonication for 20 min, with cell debris pelleted by centrifugation (5 min at $1{,}000 \times g$) between extracts. The two extracts (19 mL each) were pooled, and 10 mL water and 10 mL chloroform added to induce phase separation. After separation overnight at −20 °C, the lower phase was concentrated under N_2. Extracts were filtered over silica gel and lipids eluted with 8:2 dichloromethane:ethyl acetate. Lipid extracts were evaporated to dryness to determine yield and 1 mg of lipid reacted with 40 μL N,O-bis(trimethylsilyl)trifluoroacetamide + 1% trimethylchlorosilane and 40 μL pyridine at 70 °C for 60 min to produce trimethylsilyl derivatives. Derivatized lipid extracts were analyzed by gas chromatography-mass spectrometry on a 7890A GC/5975C MSD system (Agilent). The derivatized extract (1 μL) was injected onto a DB-1 or DB-5 column (0.250 mm ID, 0.25-μm film, 30-m length; Agilent), with He carrier flow at 1 mL/min. Analytes were ionized by electron impact at 70 eV.

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